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BIOCHEMICAL CHARACTERIZATION OF EDP208 AND ColB2 PILI

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled BIOCHEMICAL CHARACTERIZATION OF EDP208 AND ColB2 PILI submitted by GLEN D. ARMSTRONG in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

To my wife, Diane and my father-in-law, Noel

ABSTRACT

EDP208 and ColB2 pili have been purified and some of their physical and chemical properties have been compared to those of F pili. All three are conjugative-type pili. ColB2 pili are serologically related to F pili and serve as attachment sites for both F-specific RNA- and DNA- containing bacteriophage. On the other hand, EDP208 pili are not serologically related to F pili and do not attach RNA-containing, F-specific bacteriophage. However, EDP208 must share some characteristics with those of F pili, since they do attach F-specific DNA-containing bacteriophage.

An RNA-containing bacteriophage called UA-6 has been isolated which apparently attaches to EDP208 pili. Furthermore, UA-6 does not attach to F or ColB2 pili. UA-6 is about 20 nm in diameter and is made up of capsid proteins which have a molecular weight of 14,300. The phage was purified by polyethylene glycol precipitation and CsCl isopynic centrifugation. Its buoyant density in CsCl is 1.44 g/cc and the A_{260}/A_{280} ratio of pure phage is 1.70 It was hoped that this phage could be used to identify EDP208 pili in the electron microscope and to measure biological activity. Unfortunately, UA-6 attachment was very poor and it was difficult to produce high titer UA-6 lysates. The phage was not completely characterized for these reasons. However, UA-6 may be of use in identifying other plasmids which produce EDP208-like pili.

Physical studies of EDP208, F, and ColB2 pili indicate

that all three share several characteristics. They are all made up of helically arranged protein subunits called pilin. Electron microscopic studies have demonstrated that the three types of pili are resistant to disassembly by strong denaturants such as urea and guanidine hydrochloride. However, the detergent, SDS, will cause pili to disassemble into monomeric pilin. The circular dichroism studies also indicated that EDP208, F, and ColB2 pilin contain between 60-70% α-helical secondary structure.

The chemical characterization of these pili indicated several differences. The molecular weight of EDP208 and F and ColB2 pilin was estimated to be between 10,000 and 12,000 by SDS polyacrylamide gel electrophoresis. The amino acid compositions of ColB2 and F pilin were very similar and significantly different from the composition of EDP208 pilin. Nevertheless, all three pilin types possessed a high proportion of amino acids with hydrophobic side chains. EDP208 pilin lacks histidine and proline; F and ColB2 pilin lack histidine, proline, cysteine, and arginine.

Trypsin digestion of each pilin type released small peptides which were readily soluble. Tryptic peptide maps of these demonstrated sequence homologies in the peptides derived from each type of pilin.

Phosphate and carbohydrate analysis indicated the presence of phosphate and sugar in EDP208 and ColB2 pili preparations. However, further purification of the two

types of pilin by gel filtration chromatography in the presence of SDS removed all of the carbohydrate from EDP208 pilin and all but one glucose and possibly one other sugar from ColB2 pilin. This procedure was not sufficient to remove the phosphate from either of these pilins. Accordingly, column-purified EDP208 and ColB2 pilin were precipitated with acetone or extracted with chloroform-methanol. This removed all of the phosphate from EDP208 and all but one phosphate from ColB2 pilin. Acetone precipitation also failed to remove the carbohydrate from ColB2 pilin. The pilus-associated phosphate compounds which were removed by chloroform-methanol extraction were determined to be phosphatidylglycerol and phosphatidylethanolamine. Based on these investigations, it was concluded that EDP208 pilin was neither phosphorylated nor glycosylated. However, ColB2 may possess at least one covalently bound glucose moiety and a phosphate. Since F pili were not purified in sufficient quantity for phosphate and carbohydrate analysis, these studies were not extended to F pilin.

When EDP208, F, and ColB2 pilin were subjected to automated sequence analysis, all three were found to have blocked N-termini. The blocking moiety on EDP208 and ColB2 pilin was subsequently identified by ^{1}H NMR as an acetyl group. This was elucidated by isolating N-terminal peptides from pronase digests of both pilin types. The sequence of the N-terminal tripeptide from EDP208 pilin is acetyl-NH-Thr-Asp-Leu. An N-terminal hexapeptide was also

isolated from the ColB2 pilin but its sequence was not determined. However, it was concluded that the N-terminal sequence of ColB2 pilin is not the same as that of EDP208 pili.

Trypsin digestion of EDP208 pilin also produces a peptide from the N-terminal of the protein. This is probably longer than three amino acids, but its sequence has not been determined because it has not been completely separated from the other trypsin peptides.

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A_{810}	light absorbance of a solution in a 1 cm light path at 810 nm (for example)
cfu	colony forming units
col	Colicin plasmid
cpm	counts per minute
DEAE	Diethylaminoethyl
DFP	diisopropyl fluorophosphorate
DSS	sodium 2,2-dimethyl-2-silapentane-5-sulfonate
EDTA	Disodium (ethylenedinitrilo) tetraacetate
ELISA	enzyme linked immunosorbtion assay
F	F plasmid
fin	fertility inhibition
F _lac_	F plasmid containing the lactose operon of _Escherichia coli_
Hfr	high frequency of bacterial gene transfer
^{1}H NMR	proton nuclear magnetic resonance
Inc	incompatability
ma	milliamps
mCi	millicurie, 2.2×10^9 disintegrations per minute
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque forming units
ppm	parts per million
^{31}P NMR	^{31}P nuclear magnetic resonance
R	Resistance plasmid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate

SSC	saline sodium citrate
TEMED	N,N,N',N'-tetramethylenediamine
TMM	tris maleic acid minimal salts medium
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
tra	transfer
tris	tris (hydroxymethyl) amino methane
TSB	trypticase soy broth
v/v	volume per volume

CHAPTER I

INTRODUCTION

The phenomenon of bacterial conjugation is the process by which genes are transferred from one cell to another (Lederberg and Tatum, 1946). This process is mediated by small, extrachromosomal, covalently-closed, circular DNA molecules called plasmids. Contained in the plasmids are genes which encode the proteins required for conjugation.

Plasmids sometimes integrate into the main chromosome by a reversible recombinational event. In the integrated state, the transfer function of the plasmid is retained and bacterial chromosomal genes are transferred to recipient cells at a high frequency. Strains which have an integrated plasmid are called Hfr (high frequency). When an integrated plasmid becomes excised from the host chromosome, it often removes chromosomal genes with it. A classic example of such a plasmid, which is called an episome (Novick, *et al*., 1976), is F *lac*. This is an F plasmid (F for fertility) which carries the lactose genes from *Escherichia coli*.

Besides the F plasmid, which was the first to be discovered (Hayes, 1953), many other plasmids have been found. These include R plasmids, which contain antibiotic resistance genes (Watanabe, 1963), and Col plasmids which produce antibacterial agents known as colicins (Meynell *et al*., 1968).

Although the mechanism by which DNA transfer occurs is

still poorly understood, the process requires conjugative pili which are encoded by plasmid genes. These are hair-like, non-flagellar appendages, about 8.0-9.0 nm in diameter, and varying in length from 1-20 μm (Brinton, 1965). Conjugative pili provide the means whereby donor and recipient cells establish contact (Achtman and Skurray, 1977), and they may also be involved in triggering plasmid DNA metabolism during conjugation (Kingsman and Willetts, 1978). It has also been suggested that they may mediate the transfer of the DNA itself (Brinton, 1971).

Conjugative pili also serve as attachment sites for pilus-specific RNA and filamentous DNA bacteriophages (phages). The RNA-phages attach along the sides of the pili (Crawford and Gesteland, 1964; Paranchych, 1975), and DNA-phages attach to the tips of the pili (Caro and Schnös, 1966).

Clearly, a full understanding of the role that pili play in conjugation will have to include the biochemical characterization of these structures. To date, the greatest progress has been made on the characterization of F pili. Since the F plasmid was the first to be discovered and has been well characterized genetically, the following is a short summary of what is known about the F plasmid and F pili.

1. THE F PILUS

F-type pili are encoded by genes on the F plasmid

itself, many F-like R plasmids and F-like Col plasmids (Meynell and Lawn, 1967a,b; Hedges and Datta, 1971, 1972; Dennison, 1972; Hedges, 1972). As mentioned above, F pili are filamentous structures about 8.0 nm in diameter. Negatively stained pili sometimes show an axial hole about 2-3 nm in diameter indicating that F pili are tubular structures (Brinton, 1965). X-ray fiber diffraction studies support this theory (Folkhard et al., 1979). F-pili are made up of helically arranged protein subunits called pilin, which has a molecular weight of about 11,500 (Brinton, 1971; Date et al., 1977; Folkhard et al., 1979) or 10,500 (Helmuth and Achtman, 1978). F pilin is very hydrophobic in nature, containing over 50% non-polar amino acids, and lacking histidine, arginine, proline, and cysteine (Brinton, 1971; Date et al., 1977). F pilin is thought to contain one or two molecules of carbohydrate and two molecules of phosphate (Brinton, 1971; Beard and Connally, 1975), but it has never been determined whether these are covalently bound to the protein. The isoelectric pH of F pilin has been reported to be 3.8 (Valentine et al., 1969), 4.15 (Brinton, 1971), 3.5 (Beard et al., 1972a), and 3.6 (Date et al., 1977). The buoyant density for F pili has been determined to be 1.197 g/cc (Wendt et al., 1966), 1.257 g/cc (Brinton, 1971), 1.223 g/cc (Date et al., 1977), and 1.20 g/cc (Helmuth and Achtman, 1978).

F pili are remarkably resistent to denaturing reagents and show minimal loss of phage attachment ability

or of general structure in substances such as Brij 58, 8 M urea, 4 M guanidine·HCl, 10 mM deoxycholate or alkali (pH 13). However, F pili are completely dissociated in 0.1% SDS or 30 mM Sarkosyl and partially dissociated in acid (pH 1.0) or by heating at 70^{o}C (Date et al., 1977). They are also sensitive to organic solvents such as chloroform, carbon tetrachloride or benzene which destroy phage binding and cause the formation of spherical pilin aggregates (Brinton and Beer, 1967).

2. GENETICS OF THE F TRANSFER SYSTEM

The F plasmid is a covalently-closed, circular DNA molecule which has a molecular weight of 62 megadaltons. All of the genes required for conjugation are located in a 35 Kilobase region which contains the transfer (*tra*) operon (Achtman et al., 1978; Miki et al., 1978; Everett and Willetts, 1980). To date, 22 known transfer genes have been identified by genetic techniques (see figure 1). Eighteen of these are included on the transfer operon and of the first 13 *tra* genes, 12 (*tra*A-*tra*G, excluding *tra*N) are required for pilus assembly (Helmuth and Achtman, 1975). The *tra*A gene probably codes for F pilin itself (Minkley et al., 1976), but the function of the other 11 pilus gene products (p*tra*L,E,K,B,V,W,C,U,F,H,G) is unknown. As mentioned earlier, however, F pilin may be both glycosylated and phosphorylated and if this is true, then it is not inconceivable that at least some of these genes may code for enzymes required for glycosylation and phosphorylation. The *tra*S and *tra*T genes

Genetic map of the F plasmid

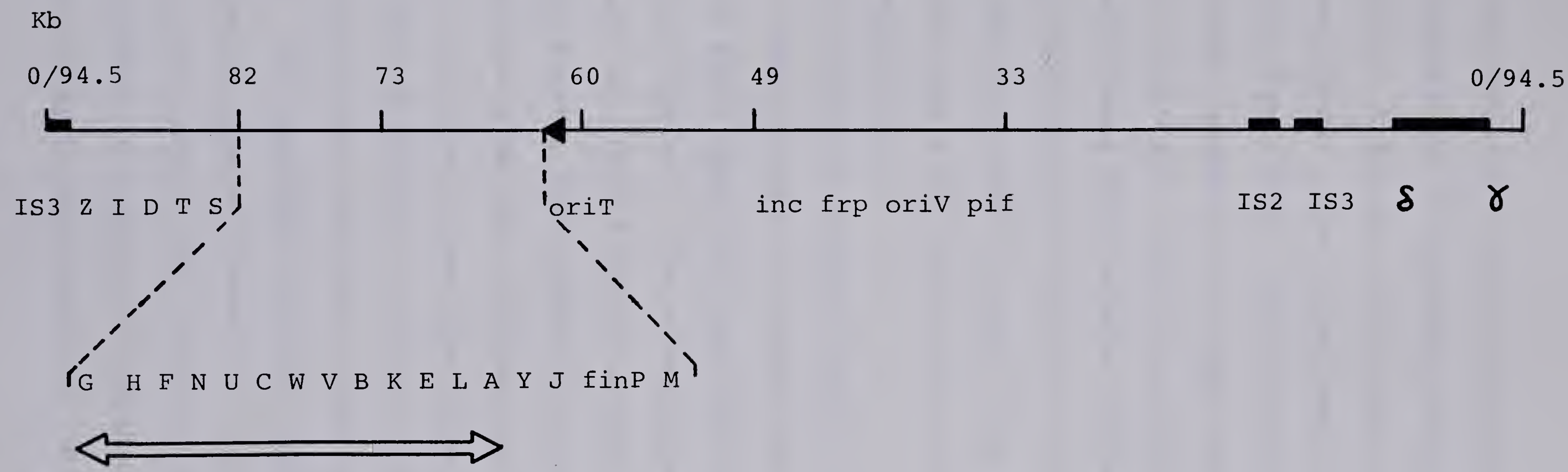


Figure 1. The transfer operon contains genes A-I inclusive. IS2, IS3, and δ-γ are insertion sequences. The numbers above the map indicate length in kilobases. This is a linear representation of the circular F plasmid.

together code for a property known as surface exclusion. This property prevents two F-containing cells from forming stable mating pairs. A protein with a molecular weight of 25,000 has been found in the outer membrane of F^+ cells (Minkley and Ippen-Ihler, 1977; Achtman et al., 1977). This protein, encoded by the traT gene, interferes with mating pair formation. The traS product functions independently and reduces DNA transfer (Achtman et al., 1977). The pif locus codes for inhibition of female-specific phage multiplication, the inc locus for incompatibility and the oriV locus is the site of initiation of vegetative replication.

In naturally occurring F-like plasmids, other than F, expression of the tra operon is positively and negatively controlled by the finO, finP, and traJ gene products (fin, fertility inhibition). The consequences of this control system are that most F-like plasmids, isolated from nature, only express their ability to transfer DNA for a short period of time after entering an F^- cell (Willetts, 1977). At other times, the transfer function is repressed. This is mediated by both of the products of the finO and finP genes (Willetts, 1972; Gasson and Willetts, 1975). The finO-finP repressor complex acts at a site, designated traO to prevent the expression of traJ (Finnegan and Willetts, 1973; Achtman, 1973). Since the traJ product is a positive regulator of the tra operon (traA to traI), repression of its synthesis

results in repression of the transfer function (Davis and Vapnek, 1976; Willetts, 1977). Most naturally occurring F-like plasmids produce both the _fin_O and _fin_P gene products. However, as seen in figure 1, F itself lacks a _fin_O gene and is, therefore, a naturally occurring derepressed mutant (McIntire and Willetts, 1978). Derepressed mutants of many other F-like plasmids have also been isolated (Cooke and Meynell, 1969; Hausmann and Clowes, 1971; Grindley _et al._, 1973, and Willetts _et al._, 1980).

The _fin_O gene product is non-specific and can combine with the _fin_P product of any F-like plasmid. Thus, if a naturally occurring F-like plasmid, producing an active _fin_O product, is co-resident with F, the transfer functions of both the plasmids is severely repressed by the functional _fin_O product. Plasmids which are capable of inhibiting the expression of F transfer are called $\underline{fin}^+$ (Willetts, 1972; Novick _et al._, 1976). Many F, Col, and R plasmids have now been isolated which are $\underline{fin}^+$ (Hedges and Datta, 1972; Hedges, 1974).

3. CLASSIFICATION OF PLASMIDS

Initially, plasmids were classified by the bacterial genes which they carried. However, this system was discarded because the same plasmid can carry many different bacterial genes, drug resistance genes, or colicin genes. Accordingly, a system was adopted to classify plasmids based on incompatibility (Inc) (Meynell _et al._, 1968).

Plasmids which cannot coexist in the same cell belong to the same Inc group (Novick _et al_., 1976). The genes which code for incompatibility are located on the plasmid itself. To date, at least 40 Inc groups have been discovered in bacteria, but only the F-like groups need to be discussed here.

There are four F-like Inc groups (FI-FV) and the plasmids in all of these are _fin_$^+$ (Hedges and Datta, 1972). Each of the four Inc groups has a "type" plasmid to which other plasmids are tested for incompatibility. The "type" plasmids for each of the Inc groups are: F, R386 (FI); R100, R1 (FII); ColB-K98 (FIII); R124 (FIV) (Novick _et al_., 1976; Hedges and Datta, 1972). These plasmids all produce F-like pili which serve as attachment sites for F-specific RNA and DNA bacteriophages (Dennison and Hedges, 1972; Paranchych, 1975). Although morphologically similar, these F-like pili are not identical because they can be distinguished serologically, and by their different efficiencies of F-specific phage attachment (Lawn and Meynell, 1970; Meynell, 1972; Willetts _et al_., 1980).

Included also with the F-like incompatibility groups is the plasmid F_o_lac_ (Inc FV). Although this plasmid has no DNA homology with F whatsoever, it does encode the production of F-like pili (Willetts _et al_., 1980). These are morphologically similar to, but serologically distinct from F pili. Interestingly, F_o_lac_

pili attach F-specific DNA, but not F-specific RNA-phages (Willetts _et_ _al_., 1980).

4. THE ROLE OF F PILI IN CONJUGATION

The fact that F pili are crucial to the process of conjugation is supported by the following evidence: (1) conjugating cells have been observed in the light microscope to be connected by an invisible bridge, presumably the F pilus (Ou and Anderson, 1970); (2) treatment of F^+ cells with pilus-specific DNA or RNA- phages blocks conjugation (Jacobson, 1972); (3) treatment of F^+ cells with pilus-specific antibody blocks conjugation (Harden and Meynell, 1972); (4) treatment of F^+ cells with cyanide, or incubation at 50°C (both treatments cause pilus retraction) blocks conjugation (Novotny and Fives-Taylor, 1974; Fives-Taylor and Novotny, 1976).

There are two theories about the function of F pili in conjugation. The first, based on light microscopic observations that conjugating cells remain separated by a significant distance, maintains that DNA is transferred through or along the pili (Brinton, 1971; Ou and Anderson, 1970). The second, proposed by Curtiss _et_ _al_ (1969) and by Marvin and Hohn (1969) maintains that F pili retract, thus bringing conjugating cells into close wall-to-wall contact where DNA transfer can occur via the retracted pilus or via a connection between the cell envelopes. This second theory is based on the observations that F pili do appear to retract into the cell membrane when F^+

cultures are treated with DNA bacteriophage, cyanide ions, or upon incubation at 50°C (Jacobson, 1972; Novotny and Fives-Taylor, 1974; Fives-Taylor and Novotny, 1976). Retraction is prevented, however, if the pili are first "coated" with RNA-phages.

Regardless of the mechanism of DNA transfer, the first stage in the conjugation process would appear to be the attachment of the pilus tip to a receptor on the recipient cell surface. It has been suggested that the major outer membrane protein, the product of the ompA gene, is the receptor in the recipient cell (Manning and Reeves, 1976; Manning et al., 1976). The result of the attachment of the pilus tip to the recipient cells results in the formation of mating aggregates consisting of between 2 and 50 cells (Achtman et al., 1978).

The second stage in conjugation is also mediated by the F pilus and involves the transmission of a signal along the pilus to trigger the synthesis or activation, in the donor cell, of the enzymes required for DNA metabolism during transfer (Everett and Willetts, 1980). These are encoded by the traY and traZ genes and cause a specific strand of DNA to be nicked at the origin of transfer oriT. The nicked strand is then transferred, 5'-end first, into the recipient cell (Vapnek and Rupp, 1970; Ohki and Tomizawa, 1968; Everett and Willetts, 1980). In the donor, the transferred strand is replaced by a newly synthesized DNA strand, although this is not required for transfer to occur (Sarathy and Siddiqi, 1973).

5. F-SPECIFIC PHAGE INFECTION

In addition to their role in conjugation, F pili also serve as receptors for F-specific RNA- and DNA-containing bacteriophage (Crawford and Gesteland, 1964; Brinton et al., 1964). The RNA-phages are icosohedral and attach to the sides of the pili; the DNA-phages are filamentous and attach to the pilus tip (Caro and Schnös, 1966). Typical RNA-phages include R17, MS2, M12, f2, and Qβ (Boedtker and Gesteland, 1975), and typical DNA-phages include fd, f1, and M13 (Meynell, 1972).

Briefly, F-specific RNA-phages are about 26 nm in diameter and contain single stranded RNA surrounded by a protein capsid made up of 180 identical coat proteins (Vasquez et al., 1966). The virion also contains a single attachment (A) protein which is non-covalently attached to the 3' end of the RNA and on the surface of the capsid (Curtiss and Krueger, 1974; Krahn et al., 1972; Wong and Paranchych, 1976).

Initiation of infection involves the attachment of the phage particle, by means of the A-protein, to the side of the F pilus. This is followed by a pilus-mediated cleavage of the A-protein into two peptides, ejection of the A-protein-RNA complex from the virion, and transfer of this to the cell surface where penetration occurs (Paranchych, 1975). Therefore, another function of the F pilus is the triggering of the phage RNA ejection step by catalysing the cleavage of the A-protein (Krahn et al.,

1972). This process is energy requiring (Knolle, 1967), and occurs only with cell-associated pili. Attachment to cell-free pili is reversible.

The filamentous DNA-phages, such as f1 and M13, attach to the tip of the F pilus. These phages contain circular single-stranded DNA molecules surrounded by a capsid 5.5 nm in diameter with a modal length of 0.7-0.9 μm (Marvin and Hohn, 1969). They also contain an attachment protein at one end (Marvin, 1966).

The early stages of DNA-phage infection closely parallel those of the RNA-phages with a few exceptions. First, the attachment process is irreversible (Tzagoloff and Pratt, 1964) and secondly, the entire virion is thought to penetrate the cell (Tzagoloff and Pratt, 1964; Trenkner *et al*., 1967; Smilowitz, 1974).

6. PURIFICATION OF F PILI

Biochemical studies on F pili have been hampered by the lack of sufficiently large amounts of F pilin, since F-containing bacteria produce only 1-2 pili/cell (Helmuth and Achtman, 1978). Also, because of their hydrophobic nature, F pili tend to adhere tenaciously to cells and cell debris, thus, further complicating their purification and reducing the final yield. Another problem with the purification of F pili is encountered in separating them from common pili (Beard *et al*., 1972b). Common pili are encoded by genes on the bacterial chromosome, have a diameter of about 7 nm and typical lengths

of 0.5-2 μm. They are usually found over the entire surface of the cells and, like conjugative pili, they are helical assemblies of identical protein subunits (MW 17,000) (Brinton, 1965). The protein (common pilin) does not contain phosphate or carbohydrate.

Several procedures have been developed for the purification of F pili. Brinton (1971) and Minkley et al. (1976) removed the pili from the cells by stirring in 30% sucrose. This procedure facilitated the separation of pili bundles. After exhaustive dialysis to remove the sucrose, the pili formed large aggregates which were easily concentrated by centrifugation. Finally, the pili were further purified in CsCl buoyant density gradients. The yield was 5 mg of 95% pure pili from 24 liters of culture. Beard et al. (1972b) published an alternate procedure which consisted of four stages: (1) blending the culture and removing the cells by centrifugation; (2) ultrafiltration to concentrate the pili; (3) CsCl density gradient centrifugation; (4) isoelectric focusing to separate the conjugative from the common pili. The yield was 3-6 mg of pili from 20 liters of culture. Other methods which result in crude F pili preparations include isoelectric precipitation at pH 4.0 (Brinton, 1965; Brinton and Beer, 1967; Valentine et al., 1969) and precipitation with polyethylene glycol and NaCl (Tomoeda et al., 1975).

Of all the conjugative pili which have now been

identified, only F pili have been investigated biochemically (Brinton, 1971; Date _et al._, 1977; Helmuth and Achtman, 1978). These studies have shown that the structural subunit of F pili (F pilin) is a hydrophobic protein which may contain two or three covalently bound carbohydrate moieties and at least two covalently bound phosphate residues. At the present time, the function of these components is unknown, but the carbohydrate may be necessary for pilus attachment to its receptor on recipient cells and/or attachment of male-specific bacteriophage. This hypothesis is based on the observation that periodate treatment of F^+ cells or purified F pili removes donor, but not recipient, ability and also reduces phage binding (Sneath and Lederberg, 1961; Tomoeda _et al._, 1975). A speculative role for the phosphate is also available. Since pilus outgrowth is energy dependent, phosphorylation of the protein may be a necessary step for pilus assembly (Novotny _et al._, 1972; O'Callaghan _et al._, 1973; Novotny and Lavin, 1971; Novotny and Fives-Taylor, 1974; Bradley, 1972a,b). Therefore, a more thorough chemical analysis of not only F, but other conjugative pili is needed to gain a better understanding of pilus function. The purpose of the present investigation was to purify enough pilin from different types of conjugative pili for comparative biochemical studies.

The two types of pili which were eventually chosen for this study were encoded by the plasmids F_o_lac_ (Inc FV)

and ColB2 (Inc FII). These are both repressed plasmids which only encode pili during short periods of time after they are transferred into F^- cells. These were chosen for two reasons. First, derepressed mutants of each type were available which overproduce their conjugative pili and thus facilitate pilus purification. The mutants were EDP208 ($F_o$lac drd), a derepressed mutant of $F_o$lac (isolated by N.S. Willetts), and ColB2 fdr, a derepressed mutant of ColB2 (isolated by Hausmann and Clowes, 1971). EDP208 encodes 15-20 pili per cell and ColB2 fdr (this will be designated ColB2) encodes 3-4 pili per cell. Second, as will be demonstrated later, ColB2 pili are closely related to F pili, chemically, serologically, and with respect to RNA-phage binding, whereas EDP208 pili are not. Therefore, it was hoped that comparative studies between these two might shed some light on the nature of RNA-phage binding sites. Finally, F pili were also purified in limited quantities and some characteristics of these pili were also determined to confirm already published results.

CHAPTER II

MATERIALS AND METHODS

1. MATERIALS

a. Bacteriophage and bacteria

(i) Bacteriophage

The RNA phages used in this study were R17, isolated by Paranchych and Graham (1962), and $Q\beta$, isolated by Nonoyama *et al*. (1963). The DNA phage was M13, isolated by Hofschneider (1963).

(ii) Bacteria

The various strains of *Escherichia coli* used in the course of this study are shown in Table 1.

All strains were preserved for long periods of time by lyophilizing a small portion of a culture grown in 5% glucose and 5% peptone at pH 7.2 and sealing the tube under vacuum. The strains were reactivated by suspending the lyophilized cells in 0.5 ml of L-broth and streaking these cells out on hard agar slants in 5.0 ml Universal bottles. The slant cultures, grown at 37°C overnight and stored at 4°C, were used to streak hard agar for single colonies. Cultures were started by transferring a single colony to 10 ml of liquid medium which was incubated with or without shaking, overnight (12-16 hours).

b. Bacterial culture media

(i) TMM (Tris (hydroxymethyl) amino methane maleic acid minimal salts medium)

The basic TMM salts solution contained the following

Table 1

Bacterial Strains

Designation	Strain	Genotype[a]	Source/Derivation
ED3873	*E.coli* K12	EDP208/JC6256[b]	N.S. Willetts
JC6256	*E.coli* K12	F^- lac trp str^s	N.S. Willetts
ED2601	*E.coli* K12	F^- lac his trp str^r spc^r T_6^r gal lys fla	W. Paranchych
ED2602	*E.coli* K12	F'lac/ED2601[c]	W. Paranchych
HB11 B/r F^- (HB11)	*E.coli* B	F^- lac fla pil	C.C. Brinton
AB257	*E.coli* K12	Hfr met str^s λ^+ [d]	E.A. Adelberg

a. The standard genotypic designations indicate inability to use lactose (lac) or galactose (gal) as carbon sources or the inability to grow without tryptophan (trp), histidine (his), lysine (lys), or methionine (met). Resistance or sensitivity to various antibodies or bacteriophage is indicated by the superscripts s or r, respectively. The antibiotics are streptomycin (str) and spectinomycin (spc) and the bacteriophage is T_6. The designations fla and pil indicate the inability to synthesize flagella or common pili.
b. Strain ED3873 is JC6256 which contains the plasmid EDP208.
c. Strain ED2602 is ED2601 which contains the plasmid F'lac. This strain can grow on lactose because of the wild type lactose operon contained on the plasmid.
d. AB257 has the bacteriophage λ genome integrated into its chromosome.

components: 0.005 M Tris; 0.05 M maleic acid; 0.043 M NaCl; 0.027 M KCl; 0.019 M NH_4Cl; 1 mM Na_2HPO_4; 1 mM NaH_2PO_4. These were dissolved in glass distilled, deionized water, and the pH was adjusted to 7.3 with HCl. The solution was sterilized by autoclaving at 126°C for 15 minutes under steam pressure of 20 lb/in^2.

To make up the complete TMM growth medium, the above solution was supplemented with glucose or glycerol (0.4%), required amino acids (0.1%) or casamino acids (0.8%), thiamine (0.02%), and $MgCl_2$ (final concentration 5 mM).

For ^{32}P-labeling, the concentration of phosphate in the minimal TMM salt solution was reduced to 0.1 mM.

(ii) <u>Trypticase soy broth (TSB)</u>

TSB (Baltimore Biological Laboratories) 15 g/l, NaCl 8 g/l, pH 7.2-7.3. Autoclaved 15 minutes, 20 lb/in^2, 126°C.

(iii) <u>Top agar</u>

TSB 30 g/l, Bacto-agar (Difco) 11 g/l. For plaque assays, the agar (stored at 4°C) was melted in a boiling water bath and dispensed into 13 x 100 mm sterile culture tubes (3 ml/tube) maintained at a temperature of 60°C in a Lab-Line multitemperature block.

(iv) <u>Hard agar (TSB agar)</u>

TSB 30 g/l, Bacto-agar 15 g/l. After autoclaving, the solution was dispensed into petri dishes (15 mm deep x 100 mm in diameter).

c. Buffers

(i) Bacteria and phage diluent

All dilutions of bacteria or phage were made with sterile solutions of SSC (Saline sodium citrate buffer) containing 0.15 M NaCl, 0.015 M sodium citrate. The pH was adjusted to the desired value with concentrated NaOH.

d. Chemicals, enzymes and reagents

All reagents were prepared from analytical grade chemicals in double-distilled water unless otherwise specified. Ultrapure sucrose was used as supplied by Schwarz Mann. CsCl and sodium dodecyl sulfate (SDS), analytical grade, were obtained from Sigma. Both mono- and dibasic sodium phosphate were obtained from Mallinckrodt Chemical Works. The chemicals used for polyacrylamide gel electrophoresis were obtained from Bio Rad (acrylamide) or from Eastman Organic Chemical Co. (Bisacrylamide, TEMED, β-mercaptoethanol). Acrylamide was recrystallized from acetone before use. DFP-treated carboxypeptidase, TPCK-treated trypsin and chymotrypsin were from Worthington Biochemicals Corp. Pronase was from Calbiochem. TRISIL and anhydrous pyridine were from the Pierce Chemical Co.

e. Radioactive materials

^{32}P for the preparation of ^{32}P-labeled EDP208 pili was obtained as $H_3{}^{32}PO_4$ (carrier free) in 0.02 M HCl from the New England Nuclear Co.

2. GROWTH OF BACTERIA

Bacterial cultures in liquid media were grown in a gyrotory-shaking water bath (New Brunswick Scientific Co.) from a 1:50 dilution of an overnight culture grown in identical medium. Maximum aeration and minimum pilus breakage was achieved by using shallow cultures (one fifth of the flask volume), shaken at 125 rpm in baffled culture flasks (Bellco Glass Co.). Under these conditions, cultures reached a cell density of 5×10^8 cells/ml in 3-4 hours using minimal media, and 2-3 hours using rich media. The density of E.coli cells in various media was determined from a standard curve constructed by plotting the absorbance at 650 nm of 5 ml of culture in a Klett-Summerson Photoelectric colorimeter versus the viable cell count (colony forming units, cfu/ml). Viable cell counts were determined by plating 0.1 ml of an appropriate dilution of the culture mixed in 3.0 ml of soft top agar on TSB hard agar petri plates. The plates were incubated overnight at 37°C and scored for bacterial colonies.

3. ISOLATION OF PHAGE FROM RAW SEWAGE

a. Fractionation of phage on sucrose gradients

Raw sewage was clarified by centrifugation at 10,000 x g for 30 minutes and the supernatant solution was mixed with an equal volume of TSB. This (0.5 ml) was layered onto 5 ml linear (5% to 40%) sucrose density gradients which were centrifuged for 30 minutes at 100,000 x g in a Beckman SW 50.1 rotor. After centrifugation, the gradients were

fractionated by collecting 0.2 ml fractions through a small hole in the bottom of the centrifuge tubes. Each fraction was then seeded with 0.1 ml of early log-phase ED3873 (JC6256/EDP208), mixed with 3 ml soft TSB top agar and plated onto TSB hard agar petri plates. The plates were examined for phage plaques after incubating 12 to 18 hours at 37°C.

b. Selection of male-specific phage

To select for male-specific RNA-containing phage, 40 plaques were picked at random from fractions 16-21 (see figure 2). These were incubated with shaking at 37°C for 18 hours in 2 ml early log-phase cultures of ED3873 (approximately 1×10^8 cells/ml). Each of the lysates was then tested for male-specificity and sensitivity to RNAse by spot testing on JC6256 (F^-) and ED3873 plus and minus pancreatic RNAse (final concentration 100 µg/ml top agar). The spot tests were performed by inoculating 0.1 ml of log-phase ED3873 into 3 ml of soft TSB top agar, layering this onto dry TSB hard agar petri plates, and applying small (about 10 µl) spots of each lysate onto the solidified agar lawns. These were incubated at 37°C, face up, until the spots were dry and for an additional 18 hours with the plates face down. In this way, up to eight mini-lysates could be tested per plate. After overnight incubation, 6 isolates, which grew only in the absence of RNAse, were chosen for further study.

c. Preparation of high titer lysates of phage UA-6

High titer lysates of one of the 6 isolates, UA-6,

were prepared by inoculating 3 ml of soft TSB top agar with 0.1 ml of log-phase ED3873 and 0.1 ml of a UA-6 mini-lysate. It was important to prepare the log-phase culture of ED3873 from freshly isolated single colonies to achieve the best efficiency of phage growth. The mixture was then layered onto TSB hard agar petri plates and incubated overnight at 37°C. The soft top agar was scraped from the plates, mixed with 3 ml TSB and homogenized with 3 drops of chloroform by vigorous shaking. After centrifugation at 10,000 x g for 10 minutes, the clear supernatant solution was removed and the agar pellet was washed by repeating the homogenization and centrifugation steps twice more. The combined supernatant solutions were stored at 5°C and used as high titer inocula in subsequent studies.

4.BACTERIAL MATINGS

Bacterial matings were carried out for 90 minutes in standing cultures at 37°C using a ratio of donor/recipient cells of 0.1. After mating, aliquots of the mixture were plated out on selective medium.

5. ABSORPTION OF PHAGE TO BACTERIA

The absorption of phage to host cells was determined by mixing phage with cells in TSB at a multiplicity of 1 phage per 10 bacteria. At timed intervals, 0.1 ml aliquots were removed and centrifuged for 3 minutes in a Beckman microfuge. The number of unattached phage remaining in the supernatant solutions was determined by plaque assay. This

was achieved by mixing 1 ml of an appropriate dilution of the supernatant solutions with 0.2 ml log-phase ED3873 and 3 ml TSB soft top agar. This was poured onto TSB hard agar petri plates and plaques were counted after overnight incubation at 37°C.

6. PURIFICATION OF UA-6 FROM CRUDE LYSATES

UA-6 was purified from crude lysates prepared by inoculating 10 ml of log-phase ED3873 with 10^4 plaque forming units (pfu) of UA-6 in 40 ml soft TSB top agar. This was layered onto 250 ml TSB hard agar in aluminum pans (27 cm x 38 cm x 1.25 cm). After overnight incubation at 37°C, the top agar was scraped from the pans and processed as described earlier (Preparation of high titer lysates).

The phage were concentrated and partially purified by the addition of 10 g polyethylene glycol 6000 (PEG, average molecular weight 6000) and 2.9 g NaCl for every 100 ml crude lysate. After standing overnight at 4°C, the PEG was pelleted by centrifugation at 7000 x g for 20 minutes. The PEG pellet, which contained the phage, was resuspended into 25 ml of SSC, layered onto the top of preformed CsCl step gradients (1.2 g/cc to 1.7 g/cc), and centrifuged for 20 hours at 20,000 rpm in a Beckman SW 27 rotor. Before use, the CsCl stock solutions used to prepare the steps gradients were filtered through millipore 0.42 μ pore size filters. The phage, which banded at a density of about 1.4 g/cc, were removed by gently sucking

the opalescent band from the gradients using a syringe and an 18 gauge needle. The preparation was dialysed against SSC at 4°C prior to further characterization.

7. PURIFICATION OF RNA FROM UA-6

To extract RNA from UA-6, purified phage (about 10^{13} pfu) were pelleted by centrifugation at 40,000 rpm for 3 hours in a Beckman SW 50.1 rotor and resuspended in 1 ml of extraction buffer (0.1 M KCl, 0.001 M EDTA, 0.001 M $MgCl_2$, and 0.01 M sodium phosphate buffer, pH 7.2). The phage protein was digested with pronase (500 µg/ml) at 37°C for 30 minutes, then one volume of buffer saturated phenol and 0.2% macaloid was added with vigorous shaking. This was centrifuged at 2000 x g for 10 minutes, and to the supernatant solution was added an additional volume of buffer-saturated phenol. The extraction procedure was repeated once more, and the combined supernatant solutions were extracted 6 times with equal volumes of diethylether. The ether was evaporated in a stream of nitrogen gas, and the RNA was precipitated by the addition of 2.5 volumes of ice cold 95% ethanol.

8. DEAE-CELLULOSE CHROMATOGRAPHY OF BASE HYDROLYSATES OF UA-6 RNA

Phenol-extracted UA-6 RNA was hydrolysed in 1 ml of 1 N KOH at 37°C for 18 hours. The alkali hydrolysate was then subjected directly to chromatography on Whatman DE81 (DEAE-cellulose) strips (1 cm x 28.5 cm). Twenty µl of the KOH hydrolysate was spotted 1 cm from the origin

of the strip and ascending chromatography was performed in 1 N acetic acid until the solvent front was 5 cm above the origin. The strip was immediately transferred, without drying, to the second solvent system (3 N formic acid) and chromatography was continued until the solvent front reached 20 cm from the origin. After drying, the nucleotide spots were visualized using an ultraviolet lamp (254 nm) and cut from the paper. One end of each paper strip containing a nucleotide spot was then sandwiched between two glass microscope slides, placed at a 15° angle in a petri dish containing 0.1 N HCl. The acid, which filled the space between the glass slides by capillary action, was allowed to elute the nucleotides by chromatography into small glass tubes, overnight, at room temperature. The eluates were dried under reduced pressure, redissolved in 0.1 M sodium phosphate buffer (pH 7.2), and the nucleotide concentrations determined spectrophotometrically. The efficiency of the procedure was determined using a standard mixture of nucleotides.

9. PURIFICATION OF EDP208, F, AND ColB2 PILI

a. Isolation of pili

Although the final stages of the purification of EDP208, F, and ColB2 pili differed somewhat, the procedures for the growth of cells and preparation of cell-free supernatant solutions that contained pili were common to each. The cells were grown by spreading 3-4 ml of fresh log-phase culture onto TSB hard agar in aluminum

pans measuring 27 cm x 38 cm x 1.25 cm. After overnight incubation, the cells were scraped from the agar and resuspended into 500 ml ice cold SSC by vigorous magnetic stirring for 2 hours. This procedure was found to remove most of the conjugative pili from the cells, leaving common pili attached. The cell suspension was passed through a stainless steel strainer (No. 48 mesh) to remove large bits of agar, then centrifuged at 10,000 x g for 20 minutes to remove cells and cell debris. The cell pellets were washed 2-3 times with SSC and the washings, plus the original supernatant solutions, were pooled (final volume 1000 ml).

b. Purification of EDP208 pili from cell-free supernatant solutions

EDP208 pili were concentrated from the cell-free supernatant solutions by the addition of 20.3 g NaCl and 20 g PEG per liter. After standing at $4^{o}C$ overnight, a white precipitate formed which was removed by centrifugation at 7000 x g for 20 minutes. The efficiency of precipitation was monitored by electron microscopy. If significant amounts of pili were observed in the 2% PEG supernatant solutions, a further 2% PEG was added and the precipitation process was repeated.

The pooled PEG pellets containing mostly pili were resuspended into 120 ml of distilled water, layered onto preformed CsCl step gradients (1.5 g/cc to 1.1 g/cc), and centrifuged for 20 hours at 20,000 rpm in a Beckman SW 27

rotor. The gelatinous white pili band (buoyant density 1.2 g/cc to 1.3 g/cc) was removed from the gradients with an 18 gauge needle and syringe and dialysed against distilled water containing a few drops of ammonium hydroxide (pH 8.0). If SDS polyacrylamide gel electrophoresis of the preparation revealed the presence of more than one protein band, the CsCl density gradient centrifugation step was repeated.

c. Purification of F and ColB2 pili

F and ColB2 pili were purified in precisely the same manner as EDP208 pili except that prior to the CsCl gradient step the PEG pellets were resuspended in 120 ml of 4 M guanidine hydrochloride. This step was included to cause dissociation of large pili aggregates.

10. PREPARATION OF ELECTRON MICROSCOPE GRIDS

Samples for electron microscopy were applied to copper grids coated with parlodion and carbon. After removal of excess sample, the grids were allowed to dry thoroughly before washing with distilled water and staining with 0.5% sodium phosphotungstate (pH 7.0). The dried grids were observed in a Philips EM 300 transmission electron microscope.

11. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS polyacrylamide gel electrophoresis (PAGE) was performed either in tube or slab gels. Tube gels (10 cm x 0.5 cm) consisted of 12.5% acrylamide, 0.8% N,N' methylene bisacrylamide, 0.07% sodium persulfate (freshly

prepared), 0.02% N,N,N',N'-tetramethylethylenediamine (TEMED), and 0.1% SDS in 0.5 M sodium phosphate buffer (pH 7.0). The protein (10-50 μg) was dissolved in 50 μl 1.0% SDS and 1.0% β-mercaptoethanol by heating in a boiling water bath for 10 seconds. Fifty μl of 60% sucrose containing 0.004% bromophenol blue was added to the cooled samples which were then layered onto the polymerized gels and electrophoresed at 5 ma/gel in 0.15 M sodium phosphate buffer (pH 7.0) containing 0.1% SDS. When the bromophenol blue dye band had migrated approximately 9 cm, the electrophoresis was terminated and the gels were stained by a modification of the procedure of Fairbanks et al. (1971). First, the gels were stained for 24 hours in a solution composed of 10% isopropanol, 10% acetic acid, and 0.05% Coomassie blue. They were then stained for an additional 24 hours in 25% isopropanol, 10% acetic acid, and 0.005% Coomassie blue. After staining, the gels were destained and stored in 10% acetic acid.

SDS PAGE on slab gels was performed by a modification of the method of Lugtenberg, et al. (1975). The slabs (1.5 mm in thickness and 16 cm in length) were prepared using stock solution 1 which contained 44 g acrylamide plus 0.8% methylene-bisacrylamide and stock solution 2 containing 30 g and 0.8 g, respectively, of these components.

The running gel contained 17.04 ml of solution 1, 1.26 ml freshly prepared sodium persulfate (10 mg/ml), 1.0 ml 10% SDS, 18.76 ml 1 M Tris-HCl (pH 8.8), 11.94 ml deionized water, and 25 μl TEMED. Before pouring, the

gel solution was filtered through Whatman No. 1 and deaerated.

The stacking gel contained 1.66 ml solution 2, 0.24 ml sodium persulfate, 0.1 ml 10% SDS, 1.25 ml 1 M Tris-HCl (pH 8.8), 7.2 ml deionized water, and 10 µl of TEMED. This solution was poured onto the top of the polymerized running gel.

The samples, containing 10-50 µg of protein, were lyophilized and redissolved in 10-20 µl sample buffer which consisted of 0.0625 ml 0.25 M Tris-HCl (pH 6.8), 0.05 ml 10% SDS, 0.05 ml 50% glycerol, 0.01 ml β-mercaptoethanol, 0.01 ml 0.5% bromophenol blue, and 0.0175 ml deionized water. These were heated for 10 seconds in a boiling water bath prior to electrophoresis. The running buffer contained 0.025 M Tris-HCl (pH 8.3), 0.19 M glycine, and 0.1% SDS. Electrophoresis was performed at a constant current of 50 ma until the bromophenol blue band was within 2 cm of the gel bottom.

12. ANTISERA PREPARATION

Antisera were prepared by injecting purified whole pili into rabbits. Approximately 100 µg of protein in 0.5 ml of sterile 0.15 M NaCl was injected into the lateral ear vein at 3-4 day intervals for 15 days. The rabbits were bled from the lateral ear vein of the opposite ear, using gentle suction, 4 to 7 days after the final injection. The blood was allowed to clot at room temperature for 4 to 6 hours before removing the serum, which

was stored at -20°C.

13. AGGLUTINATION TESTS OF EDP208 ANTISERA

The agglutination tests were performed by harvesting ED3873 and JC6256 cells from TSB agar plates and gently resuspending them at a density of about 1 x 10^9 cells per ml (determined by light scattering at 550 nm in a Gilford photometer and light source with a Beckman DU monochromator) in 0.15 M NaCl, 0.1 M sodium phosphate buffer (pH 7.2). These were then mixed, 1 to 1, with series 2-fold dilutions of antisera in the same buffer and incubated at 37°C for 1 hour. Agglutination end points were determined by noting when a serum dilution failed to cause cell aggregation greater than that observed in control suspensions containing only diluent.

14. AMINO ACID ANALYSES

Amino acid analyses were performed using a Durrum D-500 automated amino acid analyser. The values reported are average values for 24, 48, and 72 hour hydrolysis periods in constant boiling HCl containing 0.1% phenol. Hydrolysis was carried out at 110°C in evacuated sealed tubes. The values for serine and threonine were estimated by extrapolating to zero hydrolysis time. Cystine and methionine were determined as cysteic acid and methioine sulfone, after oxidizing the protein with performic acid (Moore, 1963). The value for tryptophan was determined by hydrolysing the protein in 3 N p-toluene sulfonic acid as described by Liu and

Chang (1971) or by the spectroscopic procedure of Edelhock (1967). The amount of tryptophan in standard proteins, Pseudomonas aeruginosa (PAK) pili, and lysozyme, was also determined as a control for the last two procedures.

15. DETERMINATION OF TOTAL PHOSPHATE

Inorganic phosphate was determined by the method of Chen et al. (1956). Samples suspected to contain 20 to 50 nmoles of phosphate were hydrolysed in vacuo for 72 hours in constant boiling HCl. After removing the HCl (under vacuum over NaOH pellets), the samples were dissolved in 1 ml distilled water. The color reagent was prepared by mixing 4 ml of a solution containing sulfuric and perchloric acid (18 ml concentrated sulfuric acid in 108 ml 72% perchloric acid) with 8 ml distilled water, 4 ml 2.5% ammonium molybdate, and 4 ml freshly prepared 10% ascorbic acid. One ml of the color reagent was added to the phosphate samples. The A_{810} of each sample was determined after incubation at 37^{o}C for 2 hours. Insoluble matter in some of the samples was removed by filtration through millipore (0.42 μ pore size) filters before reading the absorbance.

16. CARBOHYDRATE DETERMINATION

The amount of carbohydrate associated with purified pili was determined as glucose or galactose equivalents using the anthrone or the phenol sulfuric acid procedures (Ashwell, 1957). The assays were performed on 1 mg samples of intact and acid hydrolysed pili (4 M HCl for 2 hours at 110^{o}C in nitrogen purged, evacuated, and sealed tubes). The

limit of sensitivity of the assays was 5 µg of glucose and 2.5 µg of galactose, respectively.

17. ENZYMATIC DIGESTION OF PILIN

Before enzymatic digestion, the pili were dissociated by dissolving them in 100% formic acid. The solution was then diluted to 5% formic acid with distilled water and dried by lyophilization. In the case of EDP208 pilin, the protein was oxidized by performic acid as described by Hirs (1956). Alternately, column purified pilin was precipitated with acetone prior to digestion. The column purification and acetone precipitation procedures are described in a later section of this chapter.

Digestion was carried out in 0.1 M ammonium bicarbonate (pH 8.0) for 2-24 hours at 37°C. The ratio of protein to enzyme for trypsin digestion was 50 to 1 on a molar basis. The pronase concentration was 1% by weight of the protein.

18. TWO-DIMENSIONAL CHROMATOGRAPHY-ELECTROPHORESIS

Two-dimensional chromatography-electrophoresis was performed on plastic sheets (10 cm x 20 cm) coated with 0.1 mm of microcrystalline cellulose DEL400 (Brinkman). The samples were spotted 6 cm from one end of the sheet (anode) and 2 cm from the edge. The first dimension was ascending chromatography in n-butanol-pyridine-water-acetic acid (5:4:4:1 v/v) until the solvent front reached 1 cm from the top edge of the sheet. Electrophoresis, in the second dimension, was carried out at 500 V for 45

minutes using 8% formic acid-2% acetic acid (pH 2.1) in the electrode chambers. Under these conditions, all peptides migrate towards the cathode. The peptides were stained with the cadmium-ninhydrin reagent of Dreyer (1967). This reagent was prepared by mixing a solution of 1% ninhydrin in acetone with a solution of 5 g cadmium acetate in 250 ml acetic acid and 500 ml water (3:1 v/v). The sprayed electropherograms were heated at 60°C to detect the spots.

19. CIRCULAR DICHROISM SPECTRA

Circular dichroism studies were performed on a Cary model 60 recording spectropolarimeter with a Cary 6001 CD attachment. Samples of purified pili (0.5-1.0 mg) were prepared in 1 ml 0.1 M sodium phosphate buffer (pH 7.2) containing either 8 M urea, 6 M guanidine-HCl, 10 mM sodium deoxycholate, or 1.0% SDS. The pH of the solution was readjusted to 7.2 with HCl or NaOH, as required, and the samples were examined over the wavelength range of 200-250 nm. At a given wavelength, λ , the mean residue ellipticity, $[\theta]_\lambda$, was determined by the equation $[\theta]_\lambda = \theta_{obs} M/10 \ell c$, where θ_{obs} is the observed ellipticity in millidegrees at each wavelength, M is the mean residue molecular weight (taken as 115), ℓ is the pathlength in cm (0.0501 cm) and c is the protein concentration in milligrams per ml (protein concentration was determined by amino acid analysis and by the method of Lowry et al. (1951)). $[\theta]_\lambda$ is, therefore, expressed in degrees·cm^2.

decimole^{-1}. The apparent α-helical content of each sample was calculated using parameters given in the literature (Chen et al., 1972) (see Appendix A).

20. CHARACTERIZATION OF PHOSPHATE AND CARBOHYDRATE MOIETIES ON EDP208 AND ColB2 PILI

a. Column chromatography in the presence of SDS

Purified pili were dissolved at a concentration of 1-2 mg per ml in 50 mM Tris-HCl (pH 8.3) containing 1 mM EDTA and 1.0% SDS. The sample was applied to a Sephadex G-200 column (2.5 cm x 39 cm) equilibrated and eluted with the same buffer at a flow rate of approximately 20 ml per hour. To elute the SDS-dissolved pili, one ml fractions were collected using a Bio Rad model 1320 fraction collector. Protein in the fractions was detected by monitoring the A_{280}. The peak fractions were pooled and concentrated by lyophilization prior to further study.

b. Carbohydrate analysis

Carbohydrates were converted to free sugars by 4 M HCl hydrolysis at 110°C for 2 hours as described above. The samples were taken to dryness under vacuum and redissolved in 50% ethanol. The sugars were then identified by ascending thin layer chromatography using 0.25 mm Sil G-25 silica gel thin layer plates (Brinkman Instruments, Inc.). The solvent was freshly prepared isopropanol-ammonium hydroxide-water (7:2:1). To detect reducing sugars, the chromatograms were sprayed with the $AgNO_3$-NaOH reagent of Trevelyan et al. (1950). This involved spraying the chromatograms with a solution containing 1 ml saturated

$AgNO_3$ in 199 ml of acetone plus 5 ml water. After the $AgNO_3$-acetone spray had dried, the chromatograms were sprayed with a solution containing 2 g NaOH per 100 ml methanol. After gentle heating, the carbohydrates appeared as black spots on a buff background. The sensitivity for reducing sugars was 1 µg.

To quantitate pilin sugars, dried samples were trimethylsilylated with Trisil reagent (Pierce Chemical Co.) according to the manufacturer's directions, and the derivatized sugars were subjected to gas chromatography on a Hewlett Packard 5710 gas chromatograph equipped with programmable temperature control and a flame ionization detector. The glass column was packed with 3% OV-1 on 100/120 Supelcoport (Supelco Inc., California) and the sugars were resolved from each other by increasing the column temperature from $100^{o}C$ to $230^{o}C$ at a rate of $8^{o}C$ per minute. The helium flow rate was 60 ml per minute and the detector attenuation was 320 times. Under these conditions, 0.5 µg of sugar gave full scale deflection.

21. ^{31}P NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The pili used for ^{31}P NMR spectroscopy were dissolved in 100 mM Tris-HCl (pH 8.3), 1 mM EDTA, and 1.0% SDS. ^{31}P NMR spectra were obtained at a frequency of 109.3 MHz on a Brüker HXS-270 spectrometer interfaced with a Nicolet-1080 computer and operating in the Fourier transform mode. The spectrometer was locked on the

resonance of deuterium present as 20% D_2O in the samples. All chemical shifts were referenced to an external standard of 85% phosphoric acid and downfield shifts were given a positive sign. The spectra were recorded at 28^{o}C in 10 mm NMR tubes equipped with vortex stops. A typical setting to obtain a spectrum was pulse angle, 60^{o}, aquisition time 0.4 seconds, relaxation delay, 1.6 seconds, and sweep width, 5000 Hz. When decoupling was used, the decoupler was on during aquisition with 4 watts of power irradiating an area corresponding approximately to 3.5-6.5 ppm in a proton NMR spectrum.

22. ACETONE PRECIPITATION OF PILIN FROM SDS

Before preciptation with acetone, SDS-column purified pilin was exhaustively dialysed at room temperature against distilled water (pH 8.0 with ammonium hydroxide) and lyophilized. The dialysed protein was dissolved at a concentration of at least 2 mg per ml in distilled water, after which 4 volumes of spectral grade acetone was added. The solution was allowed to stand at room temperature for 30 minutes before collecting the precipitated pilin by centrifugation at 5000 x g for 20 minutes.

23. CHLOROFORM-METHANOL EXTRACTION OF COLUMN PURIFIED PILIN

Column purified pilin was extracted by a modification of the technique of Bligh and Dyer (1959). Samples containing 1-5 mg of protein dissolved in column buffer were mixed vigorously with 2.5 volumes of methanol, and then with 1.25

volumes of chloroform. After clarification by centrifugation at 10,000 x g for 20 minutes, the one-phase supernatant solution was decanted and the pellet was resuspended in its original volume of water and extracted a second time. The pooled supernatant solutions were vigorously mixed with 0.3 volumes of chloroform, 0.3 volumes of water, and an additional 0.6 volumes of chloroform. Centrifugation at 10,000 x g for 20 minutes resulted in the separation of the two phases. The top methanolic-water phase was separated from the lower chloroform phase by aspiration. The phospholipids, which remained in the lower phase, were concentrated by evaporating the chloroform in a stream of nitrogen gas and then separated and identified by thin layer chromatography.

24. THIN LAYER CHROMATOGRAPHY OF EXTRACTED PHOSPHOLIPIDS

Chloroform-methanol extracted lipids were separated by thin layer chromatography using the silica gel plates described above with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5) as the solvent system. The phospholipids were visualized in a sealed tank saturated with iodine vapors. The phospholipids appeared as yellow spots on a white background. The phospholipids were quantitated by scraping the spots from the thin layer plates and assaying for total inorganic phosphate following 72 hours of acid hydrolysis.

25. PREPARATION OF ^{32}P-LABELED PILI

Ten mCi of ^{32}P orthophosphate (specific activity 200 Ci/mole) was added to 100 ml early log-phase cultures

of ED3873 and JC6256, in TMM, supplemented with 0.4% glycerol, 0.8% casamino acids, and 0.8% tryptophan. These were allowed to grow for 4-6 hours, at which time the cells were removed by centrifugation at 10,000 x g for 20 minutes. The cell pellet was washed once with SSC, after which approximately 1 mg of purified, unlabeled EDP208 pili and 4.0% PEG 6000 were added to the combined supernatants. The precipitated pili were purified on CsCl step gradients as described above.

26. ISOLATION OF BLOCKED N-TERMINAL PEPTIDES FROM EDP208 AND ColB2 PILIN

To determine the nature of the blocking group on the N-terminal end of EDP208 and ColB2 pilin, 200-500 nmole of acetone-precipitated protein was digested with pronase as described previously. The soluble portion of the digest was lyophilized and redissolved in water three times to remove ammonium bicarbonate. The soluble peptides were then dissolved in 1 ml deionized water and the sample was passed through a cation exchange column containing Bio Rad AG 50W x 8 (50-100 mesh) resin. Before use, the column (0.8 cm x 7 cm) was washed with 15 ml of 1 N HCl, then extensively with deionized water until the eluate pH was 5.5 (equal to that of the wash water). The soluble pronase peptides were applied to the column and the acidic peptides were eluted with 15 ml deionized water at a flow rate of approximately one drop per second. The water eluate was lyophilized prior to characterization.

27. PARTIAL ACID HYDROLYSIS OF N-TERMINAL PEPTIDES

The N-terminal amino acid of EDP208 pilin was identified by mild acid hydrolysis of the N-terminal tripeptide isolated by the above procedure. This was achieved by hydrolysing 200 nmole of tripeptide in 0.03 N HCl at 110°C for 1 hour. The tubes were not evacuated and sealed. Hydrolysis products were separated by paper electrophoresis (pH 6.5), and the N-terminal of the unblocked tripeptide was determined by the dansylation procedure described by Needleman (1975).

28. HIGH VOLTAGE ELECTROPHORESIS

Separation of peptides by paper electrophoresis was carried out at pH 6.5. The pH 6.5 buffer contained 10% pyridine and 0.3% acetic acid. The peptides (50-500 nmole) were spotted as a 2-4 cm wide streak in the center of the paper electropherogram (Whatman No. 1). The paper was then wetted with buffer, lightly blotted, and electrophoresis was carried out at 3000 V for 45 minutes. To determine the location of each peptide, side strips were cut from the edge of the sample zone and sprayed with the cadmium-ninhydrin reagent. The developed strips were then realigned with the original paper and the regions containing the peptides were cut out. Elution of the peptides was achieved by placing one end of the strips into a trough containing the electrophoresis buffer used for separation. The other end was placed in the mouth of a small test tube held below the buffer trough. The entire apparatus was maintained in a high humidity chamber and descending chromatography (to

elute the peptides into the test tubes) was allowed to proceed for approximately 18 hours. Buffer was removed from the samples by evaporation before the peptides were subjected to further analysis.

29. GEL FILTRATION OF SOLUBLE TRYPTIC PEPTIDES

The soluble peptides released from EDP208 pilin by trypsin digestion were further purified by gel filtration on Bio Gel P2 (exclusion limit 1800). The column (1 cm x 80 cm) was packed with gel swollen in 0.1 M ammonium bicarbonate (pH 8.0). Five hundred nmole of peptides was dissolved in 0.5 ml ammonium bicarbonate and applied to the column. The flow rate was maintained at 8 ml per hour using a Pharmacia P-3 peristaltic pump. Fractions (0.5 ml volume) were collected using a Bio Rad model 1320 fraction collector.

The peptide-containing fractions were identified by removing 50 μl aliquots of every second fraction and reacting these with ninhydrin in the following manner. The fraction aliquots were first lyophilized and redissolved in water several times to remove all of the ammonia. These were then base hydrolysed using 200 μl of freshly prepared 2.5 M NaOH in open tubes in a 110°C oven. After two hours of hydrolysis, 200 μl of 30% acetic acid was added to each of the cooled tubes. This was followed by the addition of 200 μl ninhydrin reagent containing 1 g ninhydrin and 0.15 g hydrindantin dissolved in 37.5 ml of methyl cellusolve (2-methoxyethanol). Finally, 12.5 ml

4 N sodium acetate buffer (pH 5.5) was added and the tubes were heated in a boiling water bath for 15 minutes. After cooling, 1.5 ml of 50% ethanol was added before the absorbance of each fraction was determined at 570 nm.

CHAPTER III

ISOLATION OF AN EDP208-SPECIFIC RNA PHAGE

1. INTRODUCTION

Historically, one of the most important assays for conjugative pili has been the binding of pilus-specific phage. These have been used as indicators for biological activity, to identify pilus mutants, and as an unambiguous means of identifying conjugative pili by electron microscopy (Brinton, 1965, 1971; Date *et al.*, 1977; Helmuth and Achtman, 1978). Of the two types of pilus-specific phage available, filamentous DNA and spherical RNA, the latter are generally preferred for pilus studies because they are more readily visualized in association with pili by electron microscopy. Since filamentous DNA phage only attach to pilus tips, it is often difficult to identify a conjugative pilus with an attached DNA phage.

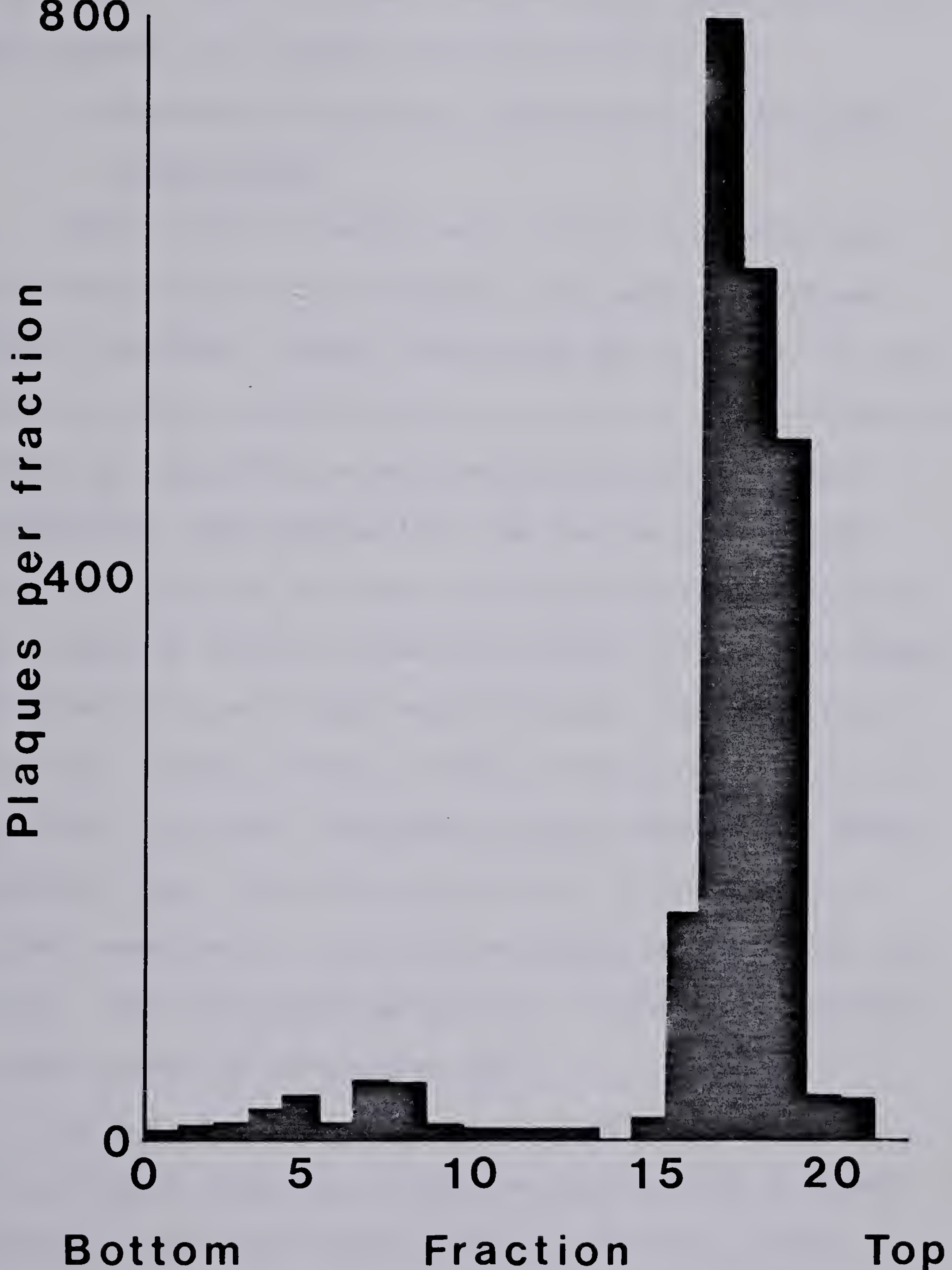
Since no EDP208-specific RNA phage has been isolated to date, an attempt was made to do so. It was hoped this would facilitate further comparitive studies between EDP208, ColB2, and F pili by providing an EDP208-specific label for electron microscopy as well as a measure for EDP208 biological activity.

2. RESULTS AND DISCUSSION

a. Fractionation of sewage phage on sucrose gradients

The results of fractionating sewage phage on sucrose gradients are shown in figure 2. It is apparent that these fall into two size classes, the majority of which belong

Figure 2. Bar graph illustrating the distribution of phage from raw sewage in 5 ml, linear sucrose density gradients. Clarified sewage was layered onto 5% to 40% sucrose gradients which were centrifuged at 100,000 x g for 30 minutes. Fractions were collected dropwise from a small hole in the bottom of the tube, seeded with 0.2 ml of log-phase ED3873, layered in TSB soft top agar onto hard agar in petri plates and incubated at 37°C for 18 hours before scoring for plaques.


800
400
0
Plaques per fraction
0
5
10
15
20
Bottom
Fraction
Top

to the lighter class which remained at the top of the sucrose gradients. Since small spherical RNA-phage were expected to be in the upper fractions (Paranchych and Graham, 1962), it was plaques from those fractions that were picked for further characterization.

b. Determination of the specificity of isolated sewage phage

Forty single plaques were picked at random from fractions 16-21 and inoculated into early log-phase ED3873 cultures. After incubation for 2 hours, no cell lysis or phage growth occurred in any of the cultures as judged by turbidity measurements and plaque assay. Accordingly, the incubation time was increased first to 8, and then to 18 hours, but still the turbidity of the cultures failed to decline and no rise in the number of infectious particles was detected. For comparison purposes, freshly grown, single plaques of R17 were inoculated into early log-phase liquid cultures of ED2602 (ED2601/F *lac*), and after two hours of incubation, a marked decrease in the culture turbidity indicated cell lysis. This was accompanied by a 10,000 fold increase in the number of infectious R17.

To test for the presence of viable phage in the forty liquid cultures, they were spot tested on fresh lawns of JC6256 (F^-) and ED3873. All forty caused clearing on the ED3873 lawns, but none showed clearing on JC6256. To screen the isolates for their sensitivity

to RNAse in the top agar, the spot tests were repeated on lawns of ED3873, both in the presence and absence of RNAse. In the presence of RNAse, 6 failed to show any clearing. These isolates, which probably contained RNA-phage (Paranchych and Graham, 1962), were selected for further characterization.

c. Selection of UA-6

Since none of the phage in the original lysates grew efficiently in TSB liquid culture, a search was made for an EDP208-specific RNA-phage which would. When all of these attempts failed, one of the 6 original isolates (UA-6) which produced the largest, clearest plaques on ED3873 was selected for further study.

d. Specificity of UA-6 for EDP208-containing cells

The results of spot testing UA-6 on the EDP208-containing strains, ED3873 and WP1000, and F-containing strains, ED2602 and AB257 (AB257 is an Hfr strain), are shown in Table 2. The phage plated only on the EDP208-containing strains, and plaque formation was prevented by the presence of 100 μg of pancreatic RNAse per ml of TSB top agar. Moreover, the F-specific RNA-phage, R17, and Qβ plated only on cells carrying the F plasmid, but, as expected, the filamentous DNA-phage, M13, plated equally well on both the F- and EDP208-containing strains. It was suggested that UA-6 is probably a pilus-specific phage by its inability to form plaques on F^- cells.

Table 2

Specificity of Phage UA-6

Phage	ED3873		WP1000[b]		ED2602 (F^+)		AB257 (Hfr)		JC6256 (F^-)	
Spotted[a]	-RNAse	+RNAse[c]	-RNAse	+RNAse	-RNAse	+RNAse	-RNAse	+RNAse	-RNAse	+RNAse
UA-6	++[d]	-	++	-	-	-	-	-	-	-
R17	-	-	-	-	++	-	++	-	-	-
Qβ	-	-	-	-	++	-	++	-	-	-
M13	++	++	++	++	++	++	++	++	-	-

a. Approximately 10^7 plaque forming units in 0.01 ml of L-broth was spotted onto a freshly seeded lawn of the appropriate indicator strain and the petri plates were examined for evidence of lysis after incubation for 8-12 hours at 37°C.

b. Strain WP1000 was constructed by mating ED3873 with ED2601, as described under Bacterial matings, and selecting for Lac^+, Str^r colonies.

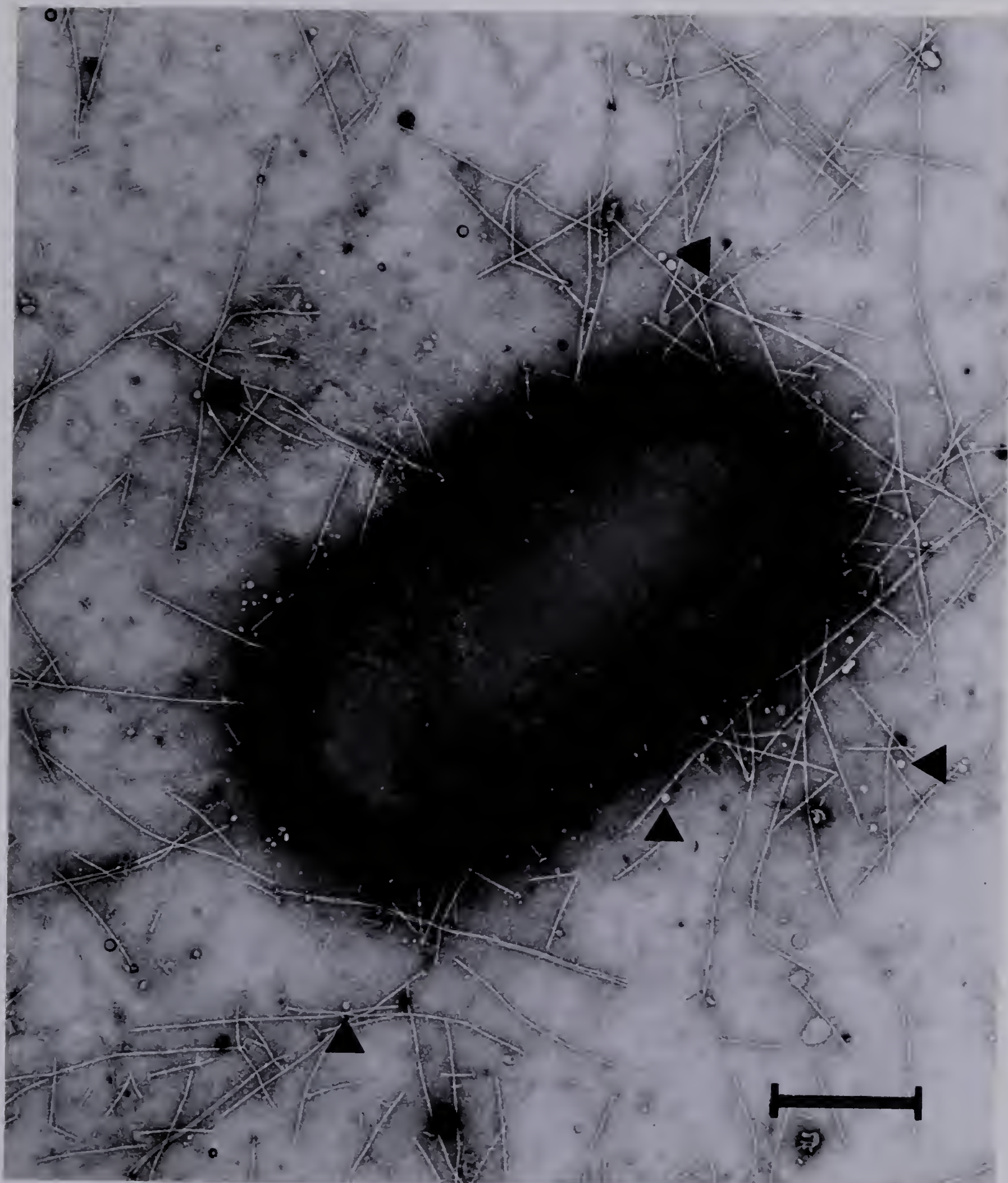
c. Pancreatic RNAse (100 μg/ml of top agar).

d. ++, clear lysis; -, no lysis.

e. <u>Absorption of UA-6 phage to ED3873</u>

The preceding investigation demonstrated that UA-6 specifically infects EDP208-containing cells. However, experiments such as these do not show whether UA-6 infection is promoted by attachment of the phage to EDP208 pili. Accordingly, the following investigations were performed to elucidate the attachment sites for UA-6. Electron microscopic examination of ED3873 cultures to which UA-6 were added revealed many pilus-attached and free phage (see figure 3). Since high titer UA-6 lysates ($> 10^{14}$ pfu/ml) could not be prepared, the evidence that UA-6 does attach to EDP208 pili was far from convincing. The low phage concentration meant that the pili were not heavily coated with phage particles as observed when R17 bind to F pili. However, evidence suggesting that UA-6 does attach to EDP208 pili was provided by the observation that anti-EDP208 pilus antiserum inhibited UA-6 infection of EDP208-containing cells. This was determined by adding aliquots of 2-fold diluted anti-EDP208 antiserum to the phage-bacteria plating mixture 10 minutes before the addition of TSB soft top agar. The plating mixture contained approximately 200 pfu of UA-6. No plaques were observed at antisera dilutions less than 256 times, whereas control antisera had no effect on phage growth. The preparation of EDP208 pili and anti-EDP208 pilus antisera is described in Materials and Methods and will be discussed later in this thesis.

Figure 3. Electron micrograph of UA-6 (arrows) and an ED3873 cell which produces EDP208 pili (many of which have become detached from the cells and are visible in the background). Both free and pilus-attached phage can be seen in the micrograph. The preparation was stained with 0.5% sodium phosphotungstate. The magnification bar represents 0.5 μm.



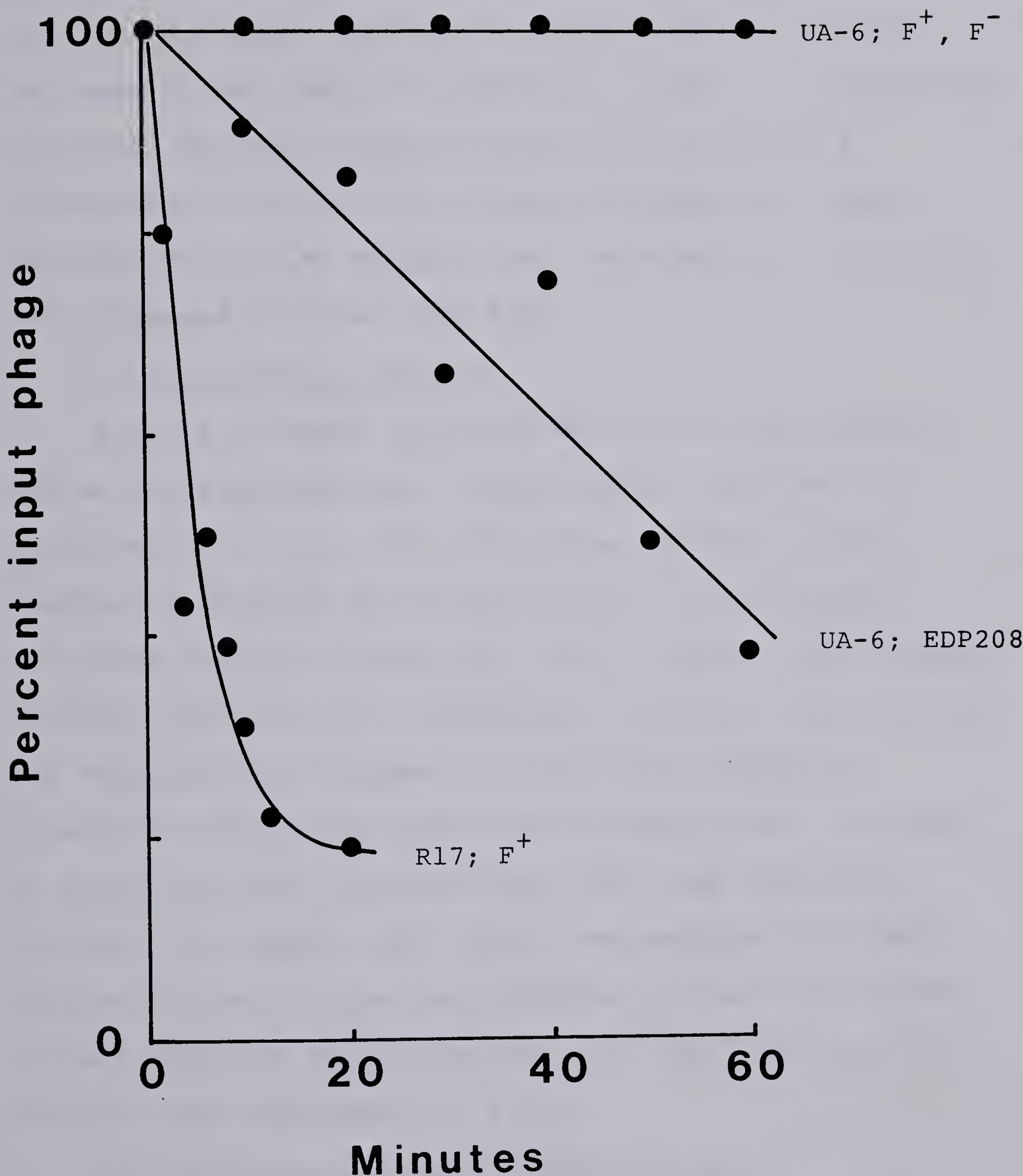
When the absorption of UA-6 to ED3873 was determined by binding assays, UA-6 was found to bind to its host much less efficiently ($K = 1.7 \times 10^{-10}$ $ml \cdot min^{-1}$) than R17 to its host ($K = 3.0 \times 10^{-9}$ $ml \cdot min^{-1}$) (see figure 4). It was also observed that UA-6 was unable to bind to plasmidless cells (JC6256) or to F^{+} cells (ED2602). This evidence further suggests that UA-6 attach to EDP208 pili but that the binding is weaker than that of R17 to F pili.

Since the major reason for isolating UA-6 was to use it as a marker for the enumeration (by electron microscopy) of EDP208 pili and as a measure of their biological activity, the poor binding of UA-6 to EDP208 pili was rather disappointing. Accordingly, investigations were initiated to determine whether UA-6 binding could be improved by altering the binding conditions. Such investigations involved varying the kinds and concentrations of divalent cations, the ionic strength of the medium, and the temperature, but none of these alternatives caused significant improvement in phage binding as judged by electron microscopy and phage growth in liquid culture.

f. Growth of UA-6 on ED3873

To examine the growth of UA-6 in liquid cultures of ED3873, UA-6 and ED3873 were mixed at different multiplicities of phage to cells. At phage-to-cell ratios less than one, no significant increase in the phage titer was detected. However, at phage-to-cell

Figure 4. Absorption of UA-6 phage to ED3873 (EDP208), ED2602 (F), and JC6256 (F^-). Phage and bacteria were mixed in TSB at a multiplicity of 1:10. At the indicated times, 0.1 ml aliquots were removed from the cultures and centrifuged. The phage remaining in the supernatant solutions were enumerated by plaque assay. For comparison purposes, the absorption of R17 to ED2602 (F) is also shown.


100
0
Percent input phage
UA-6; F+, F−
UA-6; EDP208
R17; F+
0
20
40
60
Minutes

ratios of ten or greater, the number of infectious phage increased by about 3 fold after about 2 hours of incubation (see figure 5). This increase was not substantially improved with prolonged incubation of up to 18 hours. In fact, prolonged incubation actually led to a slight decrease in the number of infectious phage. For comparison purposes, the time between infection of R17 into F-containing cells and the release of progeny R17 phage amounted to only 30 minutes, and the number of infectious R17 increased by about 1000 fold.

g. Purification of UA-6

Initial attempts to purify UA-6 from crude lysates led to the discovery that two procedures used for the purification of most other RNA-phage, methanol precipitation (Paranchych and Graham, 1962) or the liquid two-phase system of Albertson (1967), quickly led to significant losses of UA-6 infectivity. However, this problem was subsequently overcome by either centrifugation onto CsCl cushions or by PEG 6000 precipitation followed by centrifugation into preformed CsCl step gradients. Of these two methods, the latter was employed routinely because it was the most reproducible and gave the highest overall yield of phage particles (10^{13}-10^{14} pfu or 56% of total starting number of pfu's).

h. Electron microscopy of purified UA-6

Figure 6 shows an electron micrograph of purified UA-6 and reveals spherical particles roughly 20 nm in

Figure 5. Growth of UA-6 on ED3873 in TSB. Approximately 10 phage were added per ED3873 cell, and the culture was shaken at 37^{o}C. At the indicated times, samples were taken and serially diluted to determine the number of infectious phage by the plaque assay.

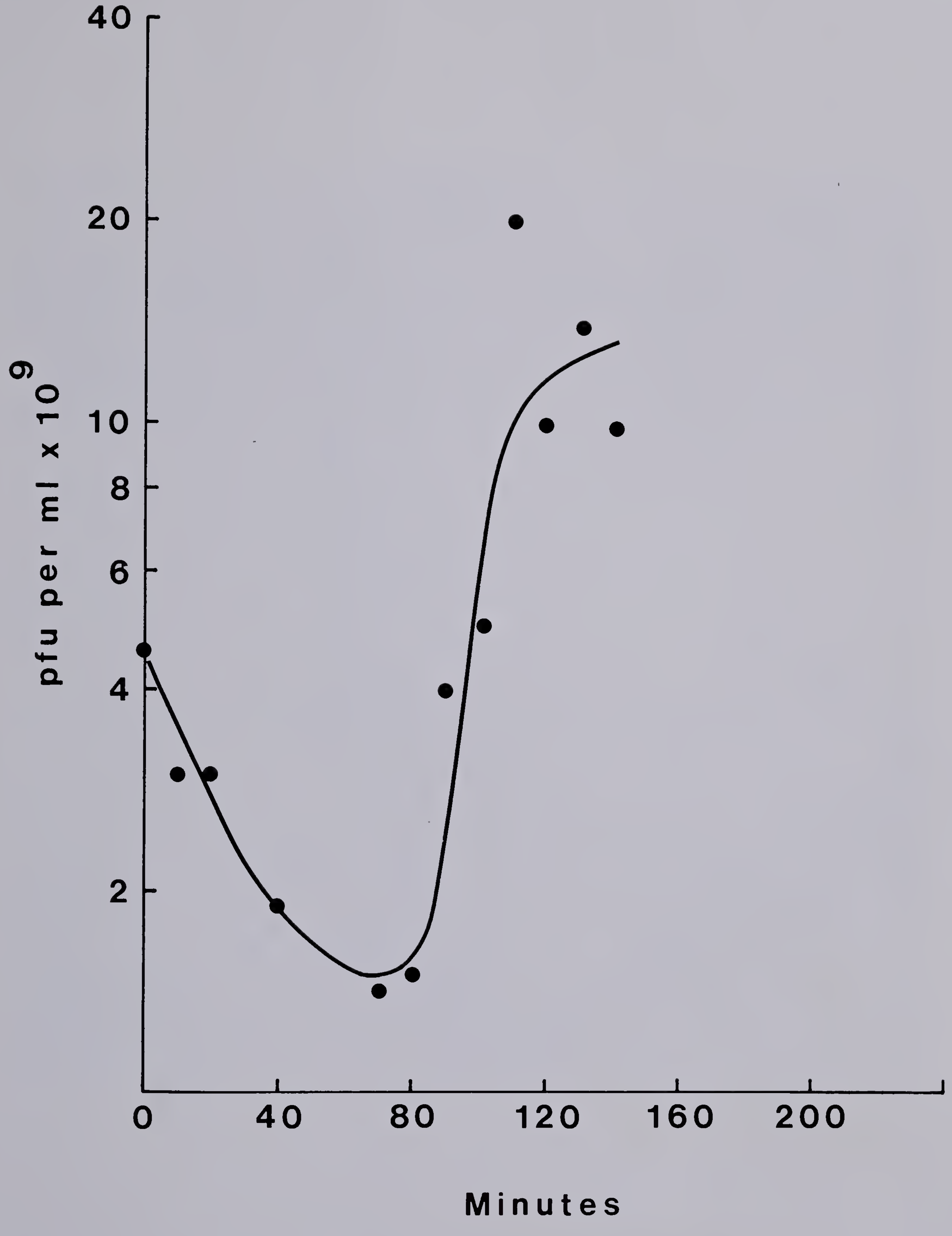
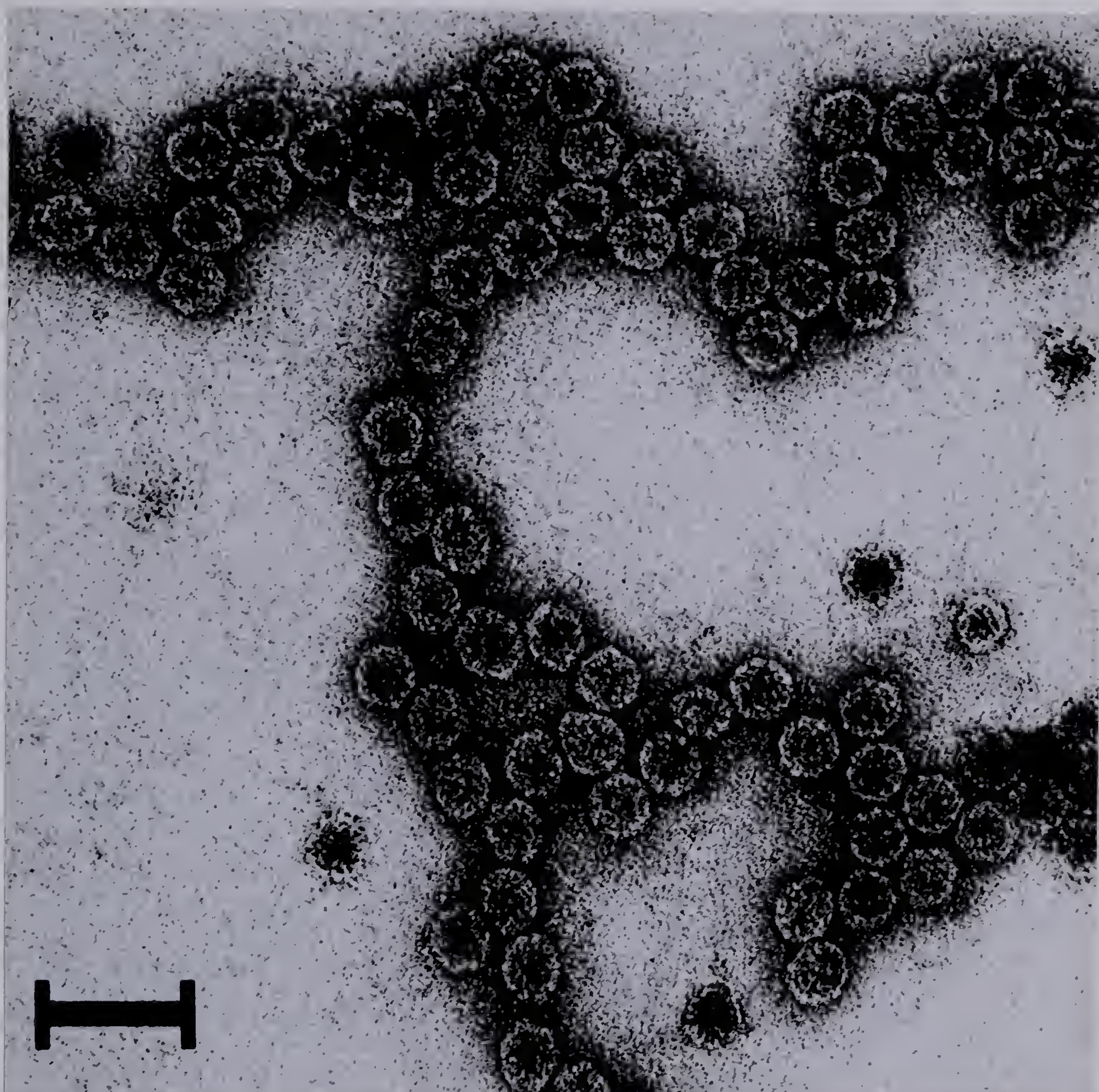

40
20
10
8
6
4
2
pfu per ml x 10^9
0
40
80
120
160
200
Minutes

Figure 6. Electron micrograph of UA-6 purified from crude lysates by 10% PEG precipitation and centrifugation into preformed CsCl step gradients (1.2 g/cc to 1.7 g/cc). The preparation was stained with 0.5% uranyl acetate and the magnification bar represents 0.05 μm.



diameter. This is smaller than R17 which has a capsid diameter of 26.6 nm (Fischbach, 1965).

i. Physical characterization of purified UA-6

Figure 7 shows an SDS polyacrylamide gel of purified UA-6 which contains one major band and several (at least 9) minor bands, most of which are probably contaminants. The major band, whose molecular weight was estimated to be about 14,300 (by comparison with the mobilities of standard proteins [see figure 8]), is most likely the coat protein of UA-6. This is somewhat larger than the coat protein of R17 which has a molecular weight of 13,750. One of the other minor bands may represent an attachment protein analogous to the attachment protein of R17 (MW 40,000, Steitz, 1975), but it was not possible to assign any specific band to a UA-6 attachment protein.

Figures 9 and 10 show the ultraviolet absorption spectrum and the buoyant density of UA-6 in CsCl. From the UV spectra, the A_{260}/A_{280} ratio for UA-6 was calculated to be 1.70 which is very close to that of PEG-purified R17 (1.74; Yamamoto et al., 1970), and from figure 10 it can be seen that the buoyant density of UA-6 in CsCl was 1.41 g/cc as compared to 1.43 g/cc for R17. The physical characteristics of UA-6 are summarized in Table 3.

Figure 7. SDS polyacrylamide tube gel of purified UA-6. Approximately 50 μg of protein in 1.0% SDS and 1.0% β-mercaptoethanol was electrophoresed into 12.5% polyacrylamide tube gels at 50 ma per gel. The electrophoresis buffer contained 0.1 M sodium phosphate (pH 7.2) and 0.1% SDS. The gels were stained with Coomassie blue by the method of Fairbanks _et al_. (1971) then destained and stored in 10% acetic acid.


Coat protein
MW 14,300

Figure 8. Estimation of the molecular weight of UA-6 coat protein by SDS polyacrylamide gel electrophoresis. Percent relative mobility is the distance moved by the protein as a percent of the distance moved by the bromophenol blue dye marker. The concentration of the acrylamide was 8%. The standard proteins were R17 coat protein (13,750), a dimer of the R17 coat protein (27,500), R17 A protein (40,000), and EDP208 pilin (10,000).

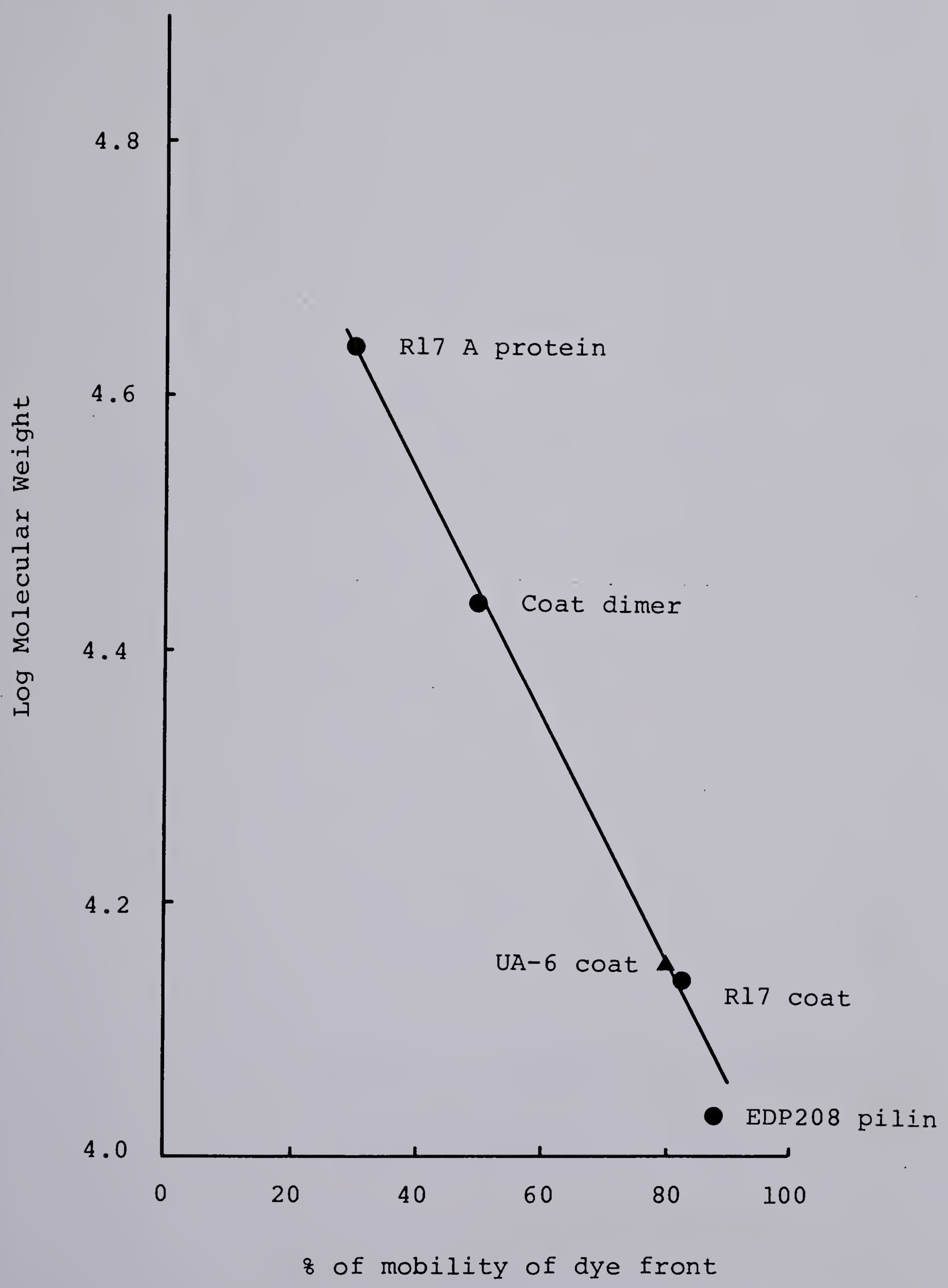

4.8
4.6
4.4
4.2
4.0
Log Molecular Weight
0
20
40
60
80
100
% of mobility of dye front
R17 A protein
Coat dimer
UA-6 coat
R17 coat
EDP208 pilin

Figure 9. Ultraviolet absorption spectrum of purified UA-6 (approximately 2×10^{13} pfu per ml in SSC). The spectrum was recorded in the wavelength range 230-340 nm on a Cary 118C recording spectrophotometer operating in the autoslit mode with a light path length of 1 cm.

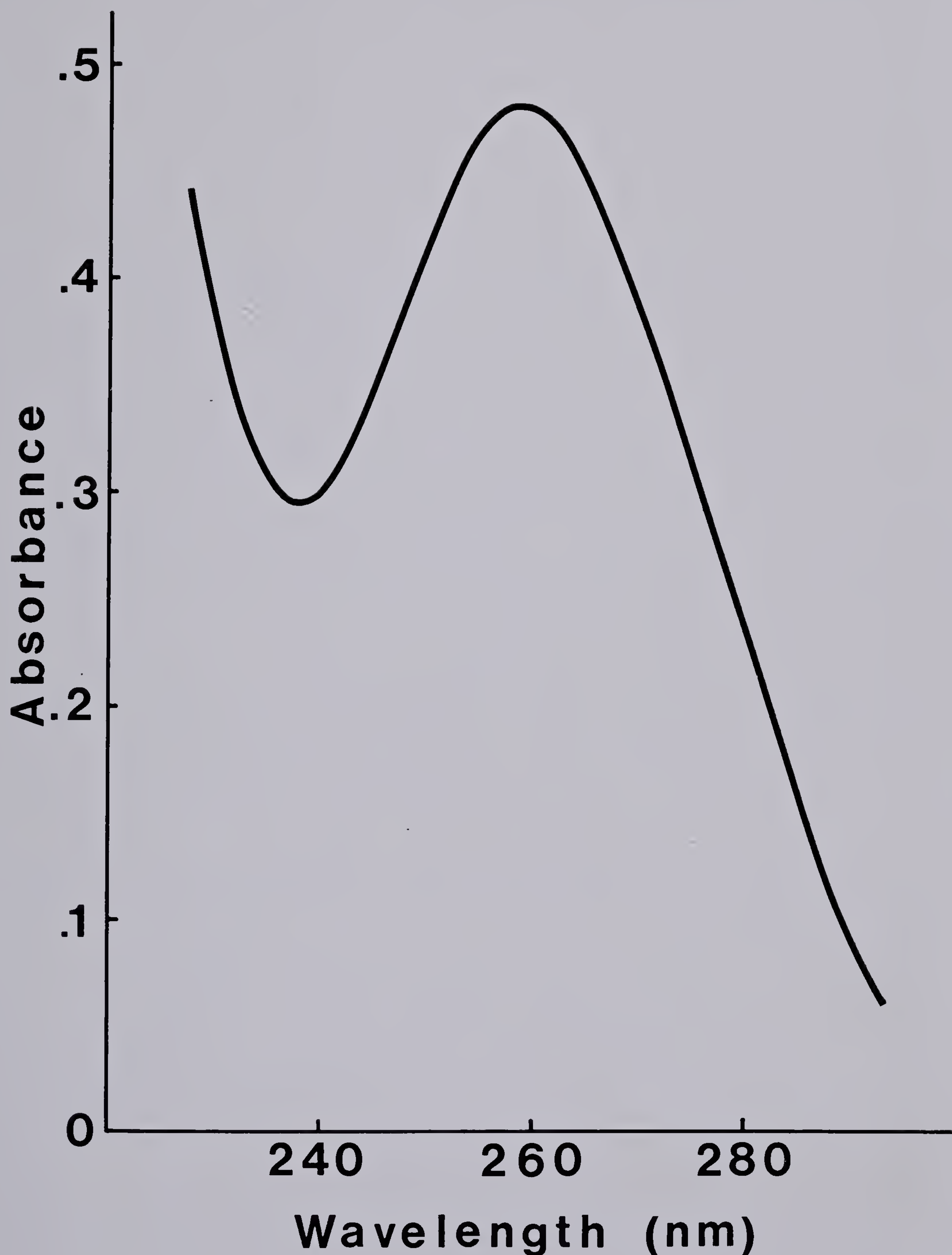
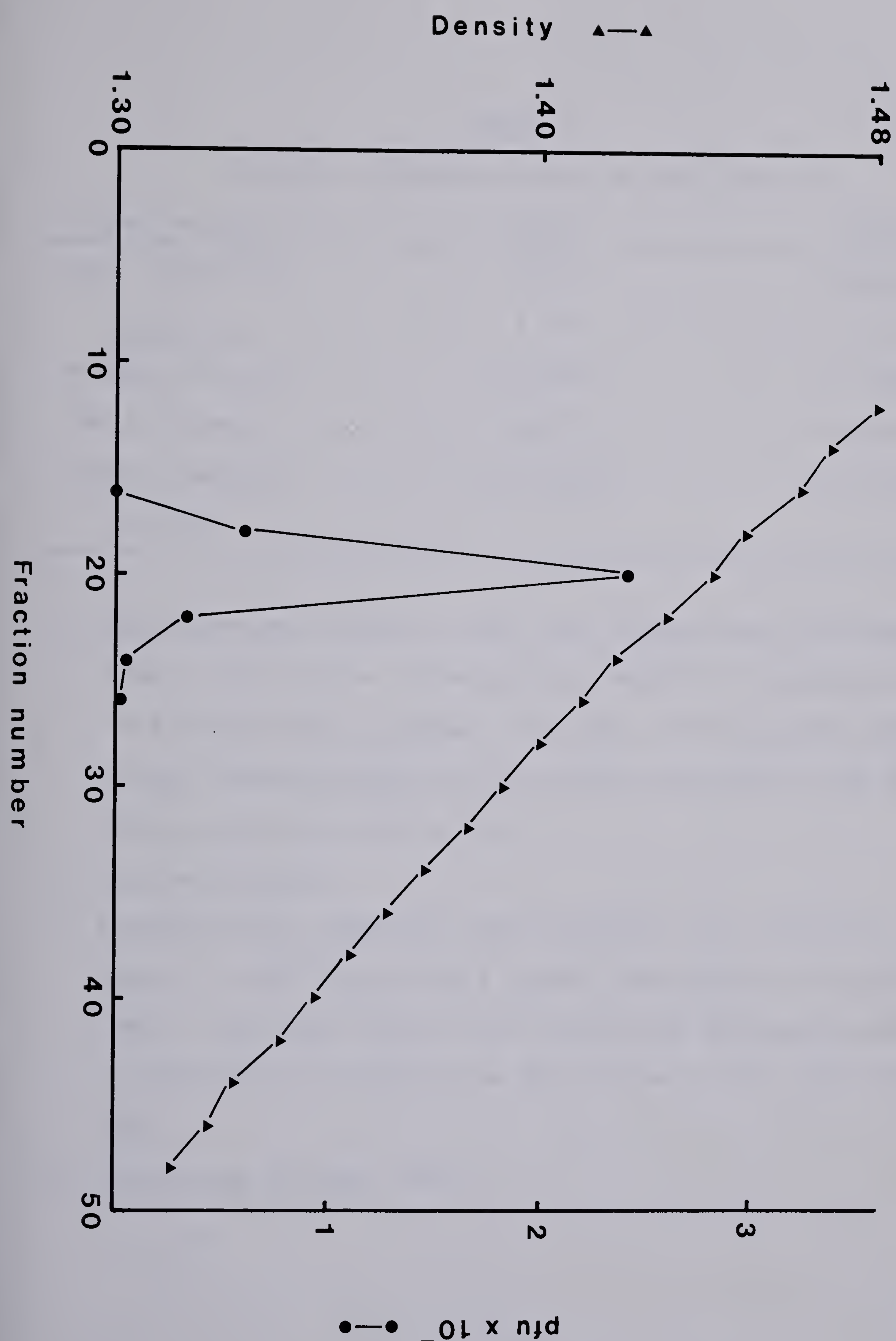

.5
.4
.3
.2
.1
0
Absorbance
240
260
280
Wavelength (nm)

Figure 10. Buoyant density of UA-6 phage in CsCl. CsCl (3.1g) was added to 5 ml of UA-6 lysate in SSC (approximately 3×10^{10} pfu), and the gradients were allowed to form at 30,000 rpm for 72 hours at $5^{\circ}C$. Fractions, collected drop-wise from the bottom of the tube, were serially diluted in SSC, and the number of viable phage in each was enumerated by the plaque assay. The CsCl density (ρ) was determined by measuring the refractive index (η) of each fraction at $25^{\circ}C$ and equating this to the density of CsCl at $5^{\circ}C$ using a graph (Frost, 1978) that related $\rho^{5^{\circ}C}$ to $\eta_D^{25^{\circ}C}$ (constructed from data obtained from the International Critical Tables).



Density
1.30
1.40
1.48
0
10
20
30
40
50
Fraction number
1
2
3
pfu x 10^2

Table 3

Physical Characteristics of UA-6 and R17

Characteristic	UA-6	R17
Size (Diameter)	20 nm	26.6 nm
A_{260}/A_{280}	1.70	1.74
MW Coat Protein	14,300[a]	13,750
MW A Protein	N.D.[b]	40,000
Buoyant Density in CsCl	1.44 g/cc[c]	1.43 g/cc[d]

a. The molecular weight of UA-6 coat protein was estimated from a plot of the log molecular weight of standard proteins versus their relative mobility. The relative mobility is the distance moved by the protein divided by the distance moved by a dye marker.

b. Not determined.

c. Determined by isopycnic centrifugation of a solution of about 3×10^{10} pfu of UA-6 in SSC containing 0.62 g/ml CsCl. The phage density was determined by plaque assay of fractions collected from the bottom of the centrifuge tube.

d. Determined by Hohn (1967).

j. <u>Isolation and base hydolysis of RNA from UA-6</u>

Purified UA-6 was assayed for the presence of RNA and DNA using the orcinol method for ribose (Brown, 1946) and the diphenylamine method for deoxyribose (Dische, 1953). Only ribose was detected. To confirm the presence of RNA in UA-6 preparations, the purified phage was extracted with phenol as described in Materials and Methods, and the extracted RNA was hydrolysed with 1 N KOH. The hydrolysate was then chromatographed on DEAE-cellulose strips using a two-phase solvent system which separates all four ribonucleotides (AMP, Rf = 0.56; GMP, Rf = 0.35; CMP, Rf = 0.77; UMP, Rf = 0.21). All of these nucleotides were subsequently identified in base hydrolysates of RNA isolated from UA-6.

The base composition of UA-6 RNA was determined by eluting the nucleotides from the paper using 0.1 N HCl and quantitating them spectroscopically. Under these conditions, greater than 90% of the standard nucleotides were recovered from the chromatograms. Of the 120 µg of UA-6 RNA hydrolysed, and spotted, 110 µg or 92% was recovered in the eluted nucleotides. The base ratio, shown in Table 4, is significantly different from that of R17 in that UA-6 appears to possess more AMP and GMP, whereas the amount of UMP is extremely low.

3. SUMMARY

The RNA-phage, UA-6, is the first RNA-phage isolated which shows specificity towards EDP208-containing cells. That

Table 4

Mole Percent of Bases in UA-6 RNA

Nucleotide	Mole Percent	
	UA-6[a]	R17[b]
AMP	34	25
GMP	35	23
CMP	21	27
UMP	10	25

a. Determined by eluting the nucleotides from an ascending DEAE-cellulose chromatogram with 0.1 N HCl and measuring their concentration spectrophotometrically. The efficiency of the procedure was monitored by using a standard mixture of mononucleotides.

b. Determined by Paranchych and Graham (1962).

UA-6 attaches, albeit poorly, to EDP208 pili is suggested by electron microscopic observations, direct binding assay to EDP208-containing cells, and by the ability of anti-EDP208 antisera to block phage growth. Furthermore, UA-6 does not grow on or attach to F-containing cells.

The phage is similar to F-specific RNA-phages in that it is spherical in shape and its capsid is probably made up of a coat protein whose molecular weight is roughly 14,300. However, its diameter (20 nm) is significantly smaller than that of R17, and the base composition of UA-6 RNA is significantly different than that of F-specific RNA-phages represented by R17.

It was hoped that the isolation of UA-6 would enable the unambiguous identification in the electron microscope of EDP208 pili on ED3873 cells. However, UA-6 attaches very poorly, and this may be one of the reasons for its poor growth in liquid culture. This made the purification of adequate amounts of UA-6 for chemical and physical analysis unprofitable and limited the phage's usefulness as a visual marker for EDP208 pili. Nevertheless, UA-6 may still be of use in identifying and grouping bacterial strains carrying plasmids of the same incompatibility group (FV) as EDP208.

CHAPTER IV

PRELIMINARY CHARACTERIZATION OF EDP208, F, AND ColB2 PILI

1. INTRODUCTION

Although conjugative pili are encoded by different kinds of plasmids and have similar morphological features, they are not identical because serological differences have been detected (Lawn and Meynell, 1970) and pilus-specific phages have been isolated (Brinton, 1965, 1971). Therefore, different types of pili may share some physical and chemical properties (those which are necessary for conjugation), and at the same time, possess differences to account for serological and phage binding differences. Clearly, the chemical characterization of different types of conjugative pili should help to elucidate those properties which are required for conjugation and phage binding.

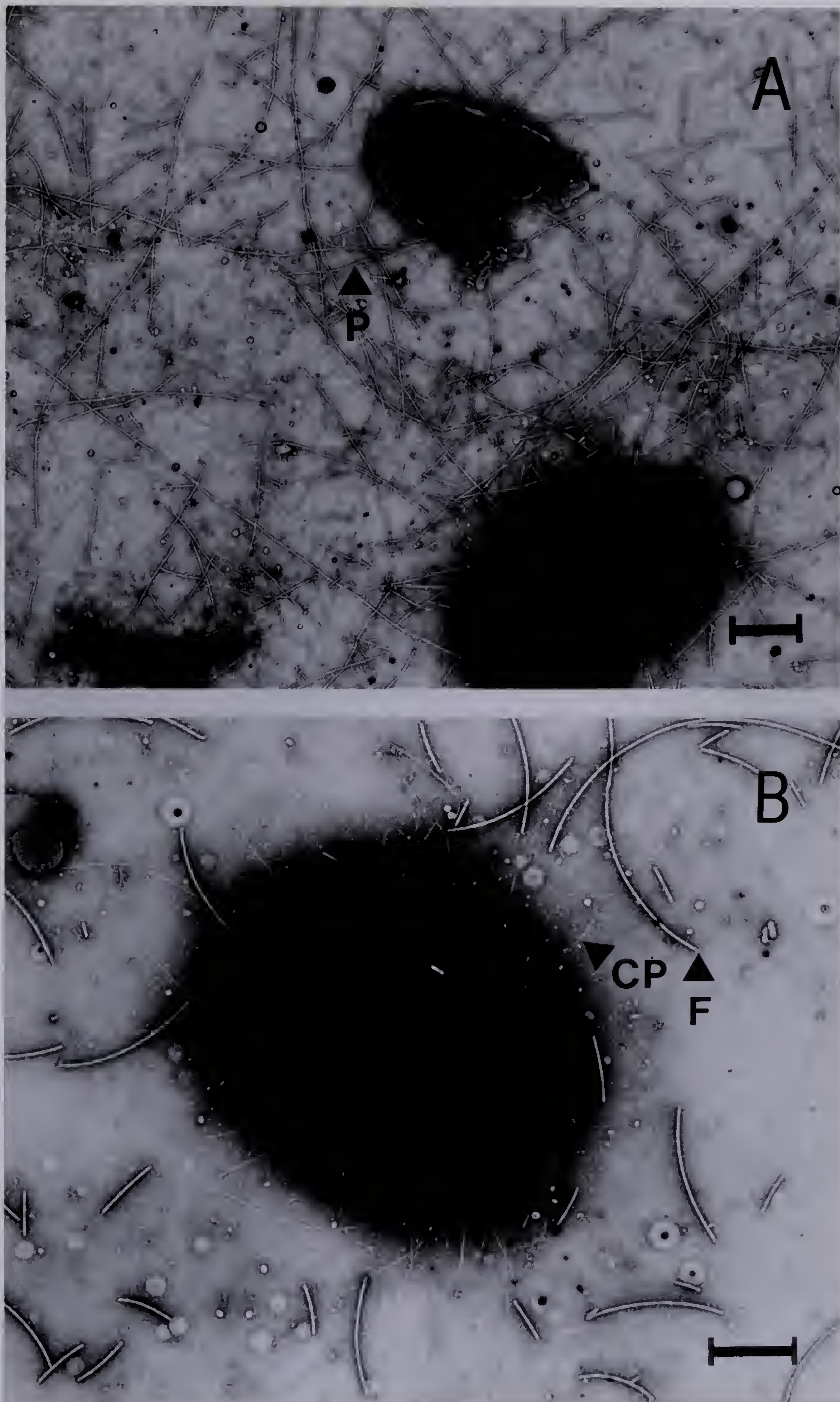
Therefore, the present studies were initiated to analyse two different F-like pilus types and to compare some of their properties with those of F pili. The chosen pili were encoded by the plasmids EDP208 (F_o lac drd [Inc FV]) and ColB2 fdr (Inc FII).

2. RESULTS AND DISCUSSION

a. Purification of EDP208 pili

An electron micrograph of ED3873 cells harvested from nutrient agar pans, and from which EDP208 pili were purified, is shown in figure 11a. These cells produce mostly conjugative-type pili (about 8.0 nm in

Figure 11. Electron micrograph of ED3873 (11a) and JC6256 (11b) cells harvested from agar plates and resuspended in SSC. The grids were stained with 0.5% sodium phosphotungstate. The ED3873 cells produce mostly EDP208 pili (P) (8.0 nm in diameter) many of which have become detached from the cells. Conversely, the JC6256 cells produce both common pili (CP) (7.0 nm in diameter) and flagella (F) (20 nm in diameter), pieces of which have also become detached from the cells. The magnification bar represents 0.5 μm.


A
P
B
CP
F

diameter) but few flagella or common pili (7.0 nm in diameter). Thus, it would appear that the EDP208 plasmid causes repression of the expression of these organelles since the F^- strain, JC6256 (shown in figure 11b), produces both common pili and flagella.

When ED3873 cells were harvested into SSC with vigorous stirring, most of the pili were detached from the cells. After centrifugation to remove the cells, the pili were concentrated by precipitation with 2.0% PEG. This procedure provided a yield of about 30-50 mg of pili per 100 g wet weight of cells. As shown in figure 12, the CsCl-purified pili are about 8.0 nm in diameter, and many possessed terminal knobs which have also been observed on F pili by Brinton (1971) and Date et al. (1977). The nature of the terminal knobs is unknown, but, their abundance seems to increase with the age of the preparations. This suggests that the pili may depolymerize from one end on aging, and that the depolymerized pilin monomers form these globular structures.

b. SDS polyacrylamide gels of purified pili

Heating in a boiling water bath for 1-5 minutes of EDP208 pili dissolved in sample buffer resulted in the precipitation of the pili material. This prevented it from penetrating into SDS gels. This problem was overcome, however, by limiting the heating time to 10 seconds. Figure 13 shows an SDS tube gel into which approximately 100 μg of EDP208 pilin has been electrophoresed. Except for a small

Figure 12. Electron micrograph of PEG precipitated, CsCl-purified EDP208 pili stained with 1.0% sodium phosphotungstate. Note the terminal knobs (K) on many of the pili. The magnification bar represents 50 nm.

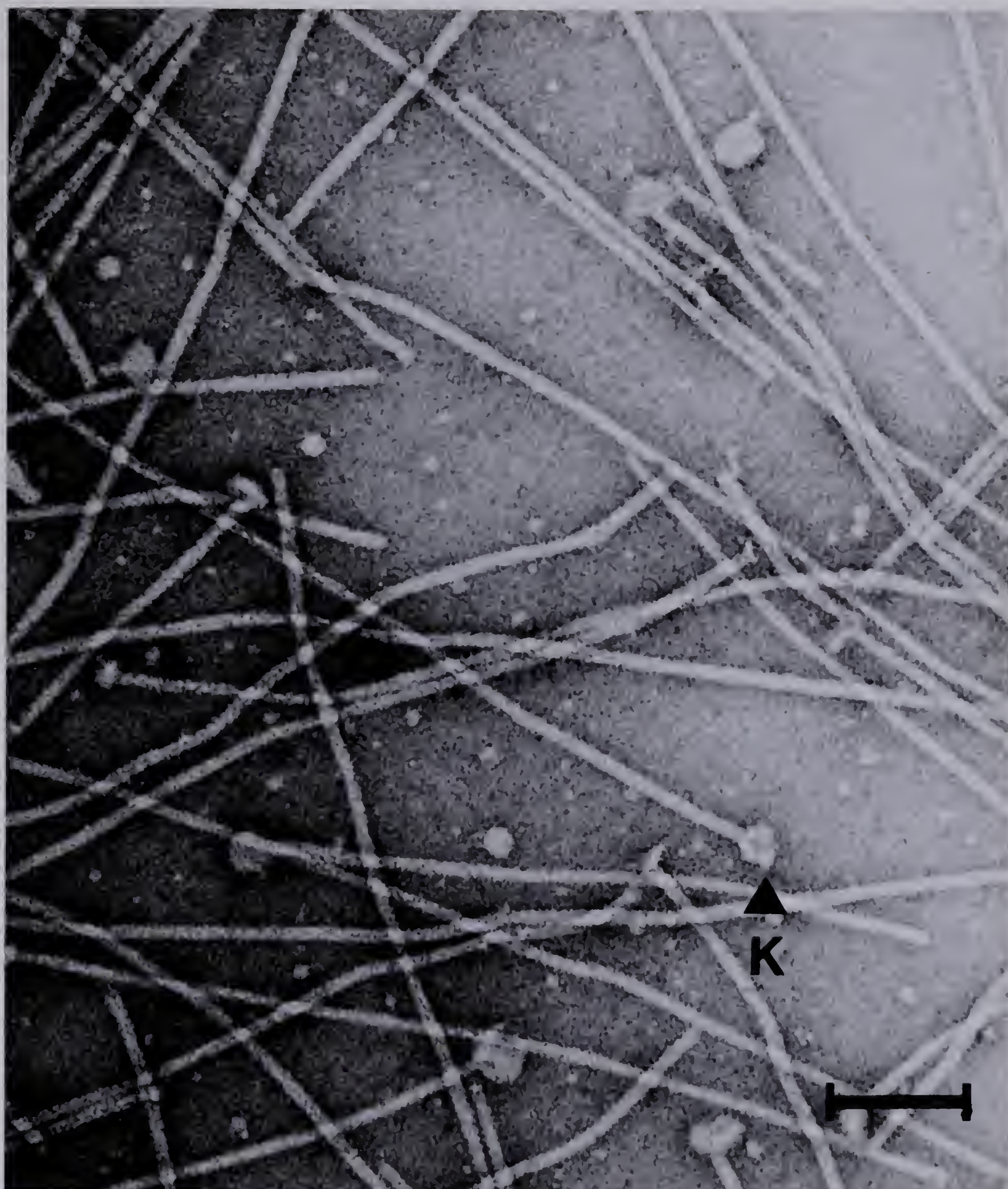

K

Figure 13. SDS polyacrylamide tube gel of EDP208 pilin. Approximately 100 µg of pilin was dissolved in 1.0% β-mercaptoethanol and 1.0% SDS by heating for 10 seconds in a boiling water bath, then electrophoresed into 12.5% tube gels at 5 ma. The electrophoresis buffer was 0.1 M sodium phosphate (pH 7.2) containing 0.1% SDS. Electrophoresis was terminated when the bromophenol dye marker had migrated 9 cm into the gel. The gels were stained by the method of Fairbanks *et al*. (1971), destained and stored in 10% acetic acid.

amount of material remaining at the top of the gel, all of the protein moved as a single band. The absence of other protein bands in the gel even when the gel was heavily overloaded suggested that EDP208 pili are composed of one type of protein subunit.

c. Determination of EDP208 pili antisera specificity

Antisera prepared to EDP208 pili were tested for their ability to agglutinate ED3873, ED2602, and JC6256 cells. As can be seen in Table 5, these were able to agglutinate only the ED3873 cells to an antiserum dilution of 1/10,240. Anti-EDP208 antisera failed to cause the agglutination of F-containing cells or F^- cells. However, since agglutination tests can sometimes be misleading due to the ability of heavily piliated cells to self agglutinate, a better assay for the presence of anti-pilus antibodies involved directly observing antibody-treated cells in the electron microscope.

The results of treating ED3873 cells with anti-EDP208 antisera are shown in figure 14a. It can be seen that ED3873 cells produce pili which were heavily coated with antibodies. For comparison, figure 14c shows ED3873 cells which have not been treated with antisera. Furthermore, anti-EDP208 antisera was not observed to bind to F pili or to common pili (Data not shown) which were sometimes observed on ED3873 cells. This evidence confirms the serological difference observed by Bradley (1977) between EDP208 and F pili. Finally, when purified EDP208 pili were

Table 5

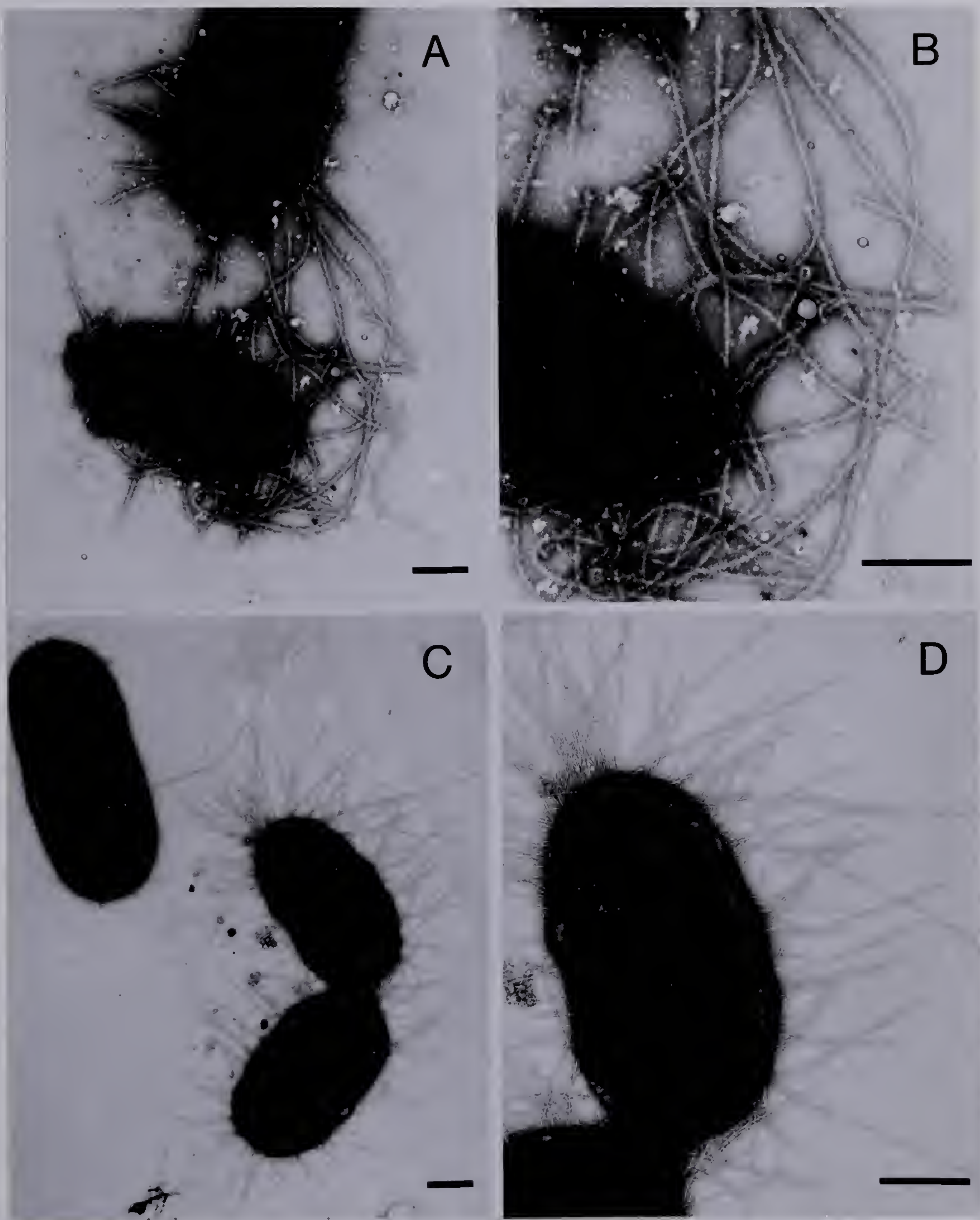
Agglutination Tests[a]

Antiserum dilution	Strain: JC6256 (F^-)	ED2602(F^+)	ED3873 (EDP208)
20	+[b]	+	++++
40	+	-	++++
80	-	-	++++
160	-	-	++++
320	-	-	+++
640	-	-	++
1280	-	-	++
2560	-	-	++
5120	-	-	+
10,240	-	-	+
20,480	-	-	-

a. Agglutination tests were performed by mixing approximately 5 x 10^8 cells with an equal volume of 2 fold serial dilutions of anti-EDP208 antiserum in 0.8% NaCl in sodium phosphate buffer (pH 7.2). Agglutination was determined after 2 hours incubation at 37°C. The end point was determined when the diluted antisera failed to cause aggregation greater than that observed when cells were mixed with diluent only.

b. (+) good agglutination, the number of +'s indicating the approximate intensity. (-) no agglutination.

Figure 14. Electron micrographs of ED3873 cells treated with anti-EDP208 antiserum (14a). ED3873 cells which were not treated with anti-EDP208 antiserum are shown in 14c. Figures 14b and 14d show higher magnification electron micrographs of EDP208 pili which have (14b) and have not (14d) been treated with anti-EDP208 antiserum. The cells were harvested from agar plates and resuspended in SSC. Samples were dried to the electron microscope grids and after washing with distilled water were inverted for 10 minutes onto a drop of 10 fold diluted anti-EDP208 antiserum on parafilm. These were covered with a small beaker to prevent evaporation. After reaction with the antiserum, the grids were again washed with water, and then stained with 0.5% sodium phosphotungstate. The magnification bars represent 0.5 μm.


A
B
C
D

treated with antisera, all of the pili in the preparations were heavily coated with antibody molecules. Therefore, the anti-EDP208 antisera could be used to identify EDP208 pili by electron microscopy.

d. Purification of F and ColB2 pili

F and ColB2 pili were purified from strains F*tra*D8/HB11 or ColB2 fdr/JC6256, respectively. The F*tra*D8 plasmid is a mutant F *lac* plasmid which overproduces F pili by about two fold, but these pili are chemically identical to wild type pili (Date *et al.*, 1977).

ColB2 and F pili are somewhat more difficult to purify than EDP208 pili because they tend to form aggregates with cell debris. Therefore, to dissociate these aggregates the PEG pellets were resuspended in 4 N guanidine hydrochloride prior to centrifugation into CsCl gradients. This treatment is made possible because F and ColB2 pili appear to remain intact in the presence of 4 N guanidine-HCl and retain almost all of their biological activity (phage binding) after removal of the guanidine (Date *et al.*, 1977). Furthermore, Date *et al.* (1977) could find no evidence for pilus disaggregation by gel filtration in the presence of guanidine hydrochloride. With this technique, 1-2 mg of pure F pili and 6-10 mg of pure ColB2 pili were obtained from 100 g wet weight of cells. SDS slab gel electrophoresis (see figure 15) demonstrated the presence of only one protein band in each preparation. For comparison purposes, EDP208 pili were also electrophoresed on

Figure 15. SDS polyacrylamide slab gel of purified ColB2 (slot A), EDP208 (slot B), and F(*tra*D8) pili (slot C). Approximately 10 µg of ColB2 and F pili and about 25 µg of EDP208 pili were dissolved in electrophoresis buffer by heating for 10 seconds in a boiling water bath. Electrophoresis was performed at 50 ma until the bromophenol blue dye band had migrated approximately 11 cm from the gel origin. The gel was then stained by the method of Fairbanks *et al*. (1971).


A
B
C

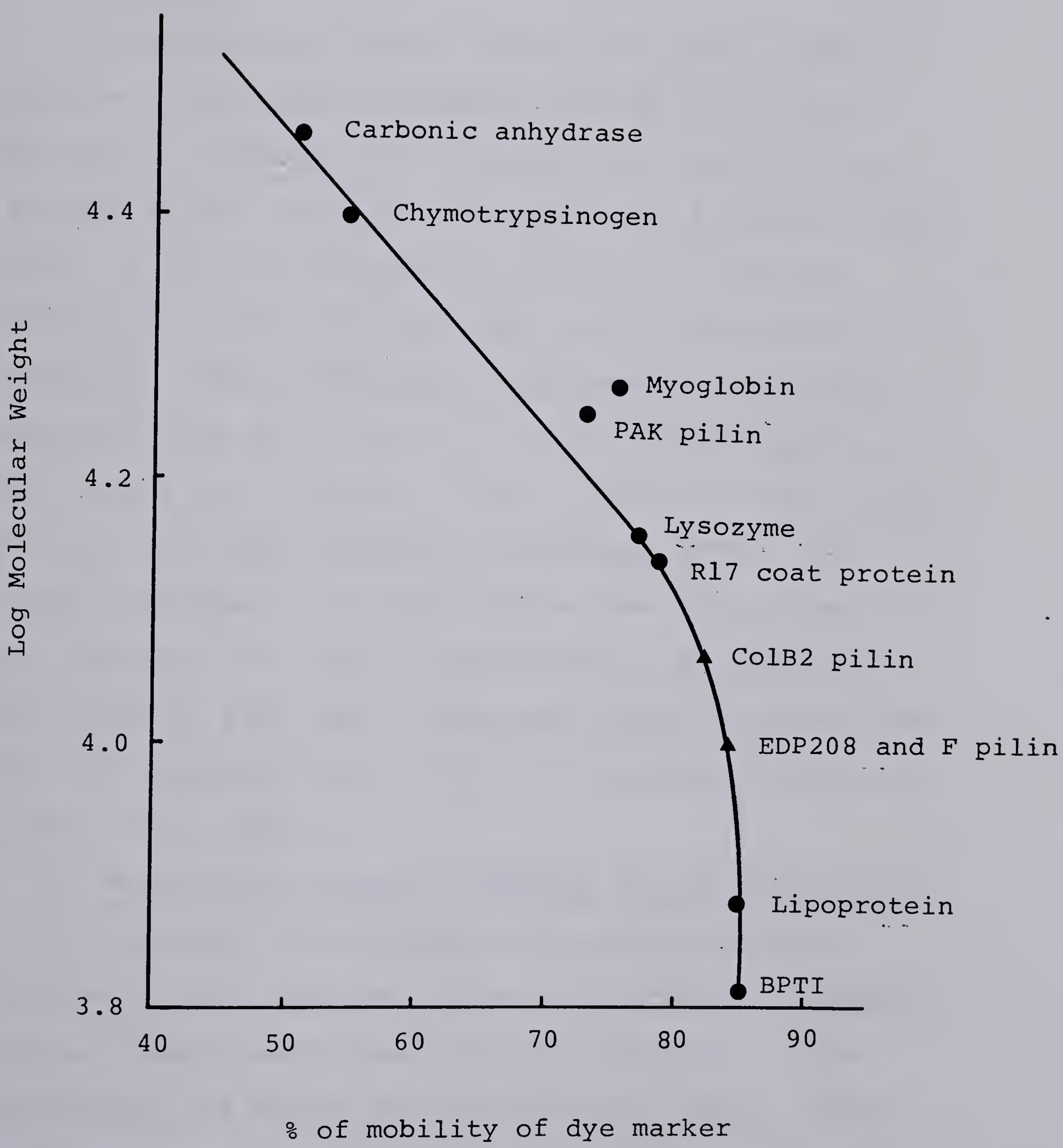
the slab gel. The molecular weight of EDP208 and F pilin was estimated to be 10,100 and that of ColB2 pilin to be 11,700 (see figure 16). Since all three pilins were consistantly found to have lower mobilities than *E.coli* lipoprotein (7500 daltons, Hirashima *et al*., 1973) and bovine pancreatic trypsin inhibitor (6500 daltons, Kassell and Laskowski, 1965), it may be concluded from these and the amino acid composition data (Table 6) that the pilin molecular weights are in the 10,000-12,000 range. However, should the pilins behave anomolously in the SDS-PAGE system, it is possible to conclude from the amino acid compositional data (Table 6) that pilin molecular weights could be in the 5000-6000 range.

Interestingly, there appeared to be more material from the EDP208 preparation remaining at the origin of the slab gels than on the origin of tube gels (shown in figure 13). Subsequent studies indicated that this material was SDS insoluble aggregates of EDP208 pilin which were formed during sample preparation for electrophoresis.

e. Serological relationship between EDP208, F, and ColB2

The serological relationships between EDP208, F, and ColB2 pili were demonstrated previously by Lawn and Meynell (1970) and by Bradley (1977). The relationships were confirmed by direct observation of antibody binding, by cell agglutination tests, and by ELISA (enzyme linked immunosorbtion assay) assays using anti-F and anti-EDP208 pili antisera (ELISA results, Paranchych, personal commun-

Figure 16. Estimation of the molecular weight of EDP208, ColB2, and F pilin by SDS polyacrylamide gel electrophoresis. Percent relative mobility is the distance moved by the protein as a percent of the distance moved by the bromophenol blue dye marker. The gel concentration was 12.5%. Standard proteins were; carbonic anhydrase (29,000), chymotrypsinogen (25,000), PAK pilin (18,100), myoglobin (17,800), lysozyme (14,700), R17 coat protein (13,750), lipoprotein (7,500), and bovine pancreatic trypsin inhibitor (BPTI, 6,500). PAK pili are produced by <u>Pseudomonas aeruginosa</u> strain K and the molecular weight of the pilin was determined by Frost (1978). Each point is the average of 6-8 determinations.



Carbonic anhydrase
Chymotrypsinogen
Myoglobin
PAK pilin
Lysozyme
R17 coat protein
ColB2 pilin
EDP208 and F pilin
Lipoprotein
BPTI
Log Molecular Weight
4.4
4.2
4.0
3.8
40
50
60
70
80
90
% of mobility of dye marker

ication). It was concluded that anti-F antiserum cross-reacted with ColB2 pili but not with EDP208 and, conversely, anti-EDP208 antiserum failed to crossreact with either F or ColB2 pili.

f. Buoyant density of EDP208, F, and ColB2 pili in CsCl

To determine the buoyant density of pili in CsCl, gradients containing approximately 100 μg of pili were subjected to isopycnic centrifugation at 35,000 rpm in a Beckman SW 50.1 rotor for 72 hours. The buoyant density values (at $5^{o}C$) were determined to be 1.232 g/cc for EDP208 pili, 1.256 g/cc for F pili, and 1.256 g/cc for ColB2 pili. These values may be compared to previously reported values for F pili of 1.197 g/cc (Wendt *et al.*, 1966), 1.257 g/cc (Brinton, 1971), 1.223 g/cc (Date *et al.*, 1977) and 1.20 g/cc (Helmuth and Achtman, 1978). The buoyant densities of the pili may be lower than those of most proteins (1.33 g/cc) possibly due to phospholipids which copurify with them. These were found in EDP208 and ColB2 pili preparations and will be discussed in the next chapter of this thesis.

g. N-terminal analysis of EDP208, F, and ColB2 pilin

To determine the N-terminal amino acid residue of the three pilins, they were reacted with dansyl chloride (1-dimethylaminonapthalene-5-sulfonyl chloride) in the presence of 1.0% SDS as described by Gray (1972). Under these conditions, the N-terminal amino acid residue of

lysozyme (lysine) was easily identified in acid hydrolysates of the dansylated protein. However, all three types of pilin failed to react with the dansyl chloride. Alternately, the three pilin proteins were subjected to automated Edman degradations in a Beckman 890B sequenator as described by Paranchych *et al*. (1978). Once again, all three pilins, suspended in the conventional Quadrol buffer, failed to react with the phenylisothiocyanate, indicating the existance of a blocked amino group on the N-terminal amino acid residue of these proteins.

h. Compositional analysis of EDP208, F, and ColB2 pili

Purified pili preparations were subjected to amino acid analysis and the results of these studies are summarized in Table 6. For comparison purposes, the amino acid analysis of F(*tra*D) pili is also included. The table indicates that F and ColB2 pilin are very similar and are significantly different from EDP208 pilin. Generally speaking, however, all three types of pilin contain a fairly high proportion (about 53%) of hydrophobic amino acids (Ala, Leu, Ile, Val, Phe, Trp, Met). All of the proteins lack histidine and proline, possibly indicating a high α-helical content in each. The F and ColB2 pilins were also found to lack arginine and cysteine, and EDP208 pilin lacked tryptophan. It was also observed that the calculated molecular weights for all three types of pilin agree with those estimated by SDS polyacrylamide gel electrophoresis. It should be noted, however, that these molecular weights

Table 6

Amino Acid Analysis of EDP208, ColB2, and F Pilin

Amino Acid	EDP208		ColB2		F		F(traD)[a]	
Lys	7.3 ± 1.06[b]	(8)[c]	8.5 ± 0.34	(9)	7.1 ± 0.37	(9)	7.6 ± 0.5	(10)
His	0.0	(0)	0.0	(0)	0.45	(0)	0.15 ± 0.05	(0)
Arg	2.0 ± 0.44	(2)	0.0	(0)	0.49	(0)	0.30 ± 0.15	(0)
Asp	9.9 ± 0.82	(10)	6.7 ± 0.71	(8)	7.5 ± 0.51	(8)	6.6 ± 0.6	(8)
Thr	13.5 ± 0.43	(14)	6.2 ± 0.23	(9)	5.2 ± 0.26	(7)	6.4 ± 0.4	(8)
Ser	2.4 ± 0.15	(2)	6.4 ± 0.50	(9)	7.8 ± 0.21	(10)	9.3 ± 0.9	(11)
Glu	2.06 ± 0.22	(2)	4.6 ± 1.00	(8)	3.5 ± 0.03	(4)	3.6 ± 0.4	(4)
Pro	0.0	(0)	0.0	(0)	0.0	(0)	0.0	(0)
Gly	10.5 ± 0.70	(11)	10.4 ± 0.65	(13)	11.9 ± 0.26	(15)	11.6 ± 0.3	(15)
Ala	9.2 ± 1.02	(10)	14.0 ± 0.45	(16)	13.2 ± 0.38	(15)	12.4 ± 0.3	(15)
½Cys	2.1 + 0.50	(2)	0.0	(0)	0.0	(0)	0.0	(0)
Val	10.4 ± 0.20	(12)	16.3 ± 0.91	(20)	18.8 ± 0.40	(23)	16.7 ± 1.7	(21)
Met	4.3 ± 0.69	(5)	6.5 ± 0.36	(8)	5.9 ± 0.47	(8)	6.9 ± 0.2	(8)
Ile	9.5 ± 0.05	(10)	3.5 ± 0.29	(4)	3.9 ± 0.12	(5)	3.1 ± 0.4	(4)
Leu	12.1 ± 0.30	(14)	8.1 ± 0.23	(9)	8.0 ± 0.19	(9)	7.3 ± 0.5	(9)
Tyr	1.6 ± 0.42	(2)	1.8 ± 0.13	(2)	1.5 ± 0.08	(2)	1.5 ± 0.2	(2)
Phe	6.0 ± 0.41	(6)	6.4 ± 0.31	(7)	5.2 ± 0.88	(6)	5.3 ± 0.3	(7)
Trp	0.0	(0)	1.0 ± 0.50	(2)	2.0 ± 1.00	(2)	1.4 ± 0.2	(2)
Total	102.8	110	100.4	124	102.4	123	100.1	124
Molecular Wt[d]	11,900		12,900		12,600		12,700	

a. From Date *et al.*, 1977

b. The mole percent values for EDP208 and ColB2 pilin are averages computed from determinations of two different samples following 24, 48, and 72 hours of acid hydrolysis. Threonine and serine were determined by extrapolation to zero time of hydrolysis. The values for valine and isoleucine are for 72 hours of hydrolysis. The values for F pilin are averages of two different samples following 48 hours of hydrolysis.

c. The numbers in brackets give the residues per pilin subunit based on the molecular weights determined by SDS polyacrylamide gel electrophoresis.

d. Molecular weight based on the amino acid composition.

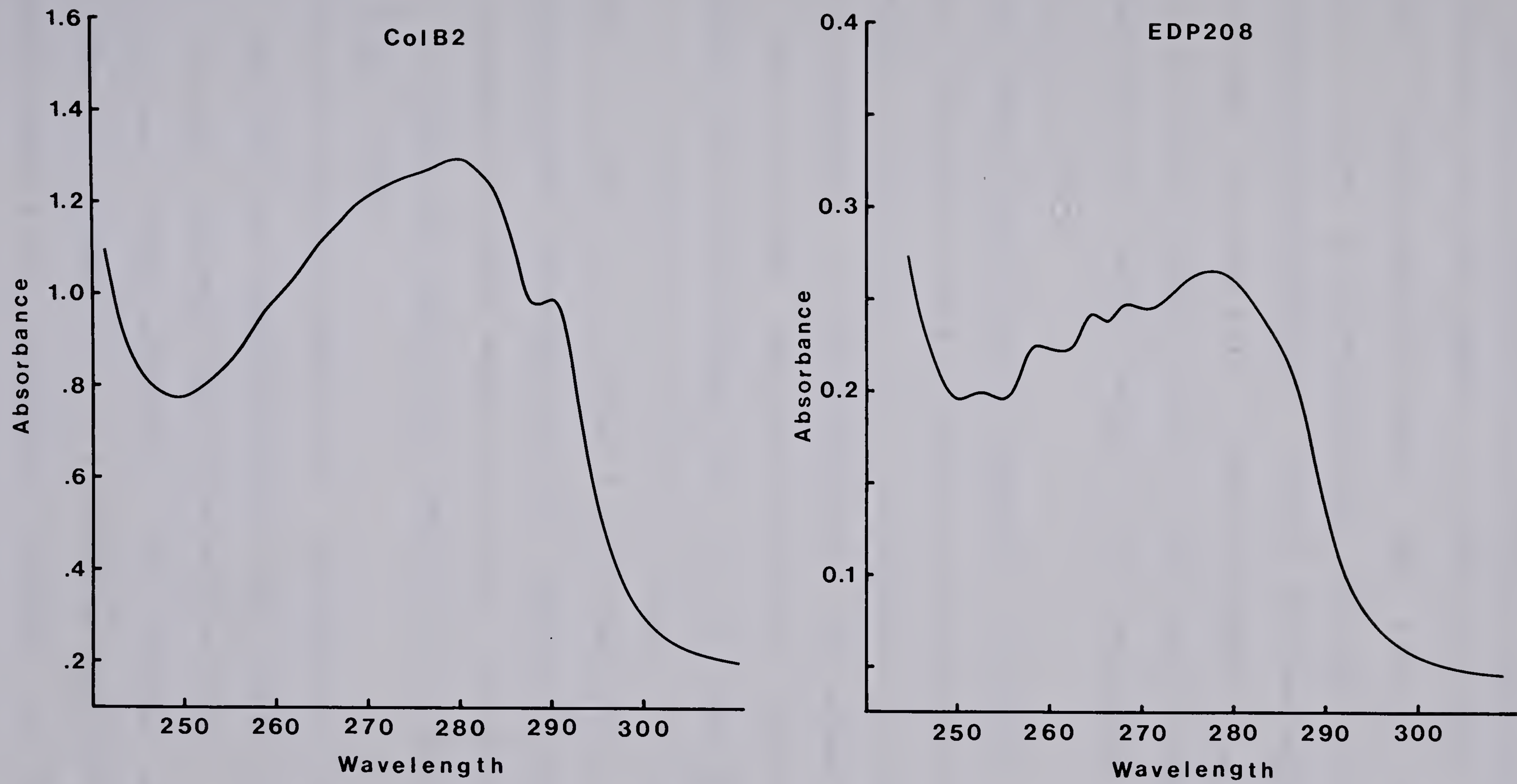
could be overestimates. Based on the amino acid compositions, the minimum molecular weight for EDP208 pilin was calculated to be 6,000 and that for ColB2 and F pilin was calculated to be 6,500 and 6,300, respectively. As mentioned earlier, the observed mobilities of EDP208, F, and ColB2 pilins in the SDS-PAGE system (figure 16) suggest their molecular weights are probably in the 10,000-12,000 range.

Phosphate and carbohydrate analysis indicated the presence of both phosphate and carbohydrate in purified pili preparations. Determination of total inorganic phosphate by the method of Chen _et al_. (1956) indicated two or possibly three phosphates per pilin in each case. Colorimetric assays using the phenol sulfuric acid or anthrone procedures indicated that all three pili types possessed 2 or 3 moles of hexose per mole of protein. These results confirmed those for F(_tra_D) of Brinton (1965) and Date _et al_. (1977). The nature of both the phosphate and carbohydrate moieties will be discussed in Chapter V.

i. Ultraviolet absorption spectra of EDP208 and ColB2 pili

Ultraviolet absorption spectra of EDP208 and ColB2 pili are shown in figure 17. The two differences to note are the absorbance of tryptophan at 290 nm in ColB2 pilin, absent from the EDP208 spectra and the phenylalanine fine structure in the EDP208 spectrum, missing from the ColB2 spectrum. The latter may be due to the masking of the phenylalanine absorbance in ColB2 pilin by that of tryptophan.

Figure 17. Ultraviolet absorption spectra of EDP208 and ColB2 pilin in the presence of 1.0% SDS. Approximately 1 mg of pilin was dissolved in 0.1 M sodium phosphate buffer (pH 7.2 $\pm$ 0.1) containing 1.0% SDS. The samples were scanned using a Cary model 118C spectrophotometer. The light path was 1 cm.



ColB2
Absorbance
1.6
1.4
1.2
1.0
.8
.6
.4
.2
250
260
270
280
290
300
Wavelength
EDP208
Absorbance
0.4
0.3
0.2
0.1
250
260
270
280
290
300
Wavelength

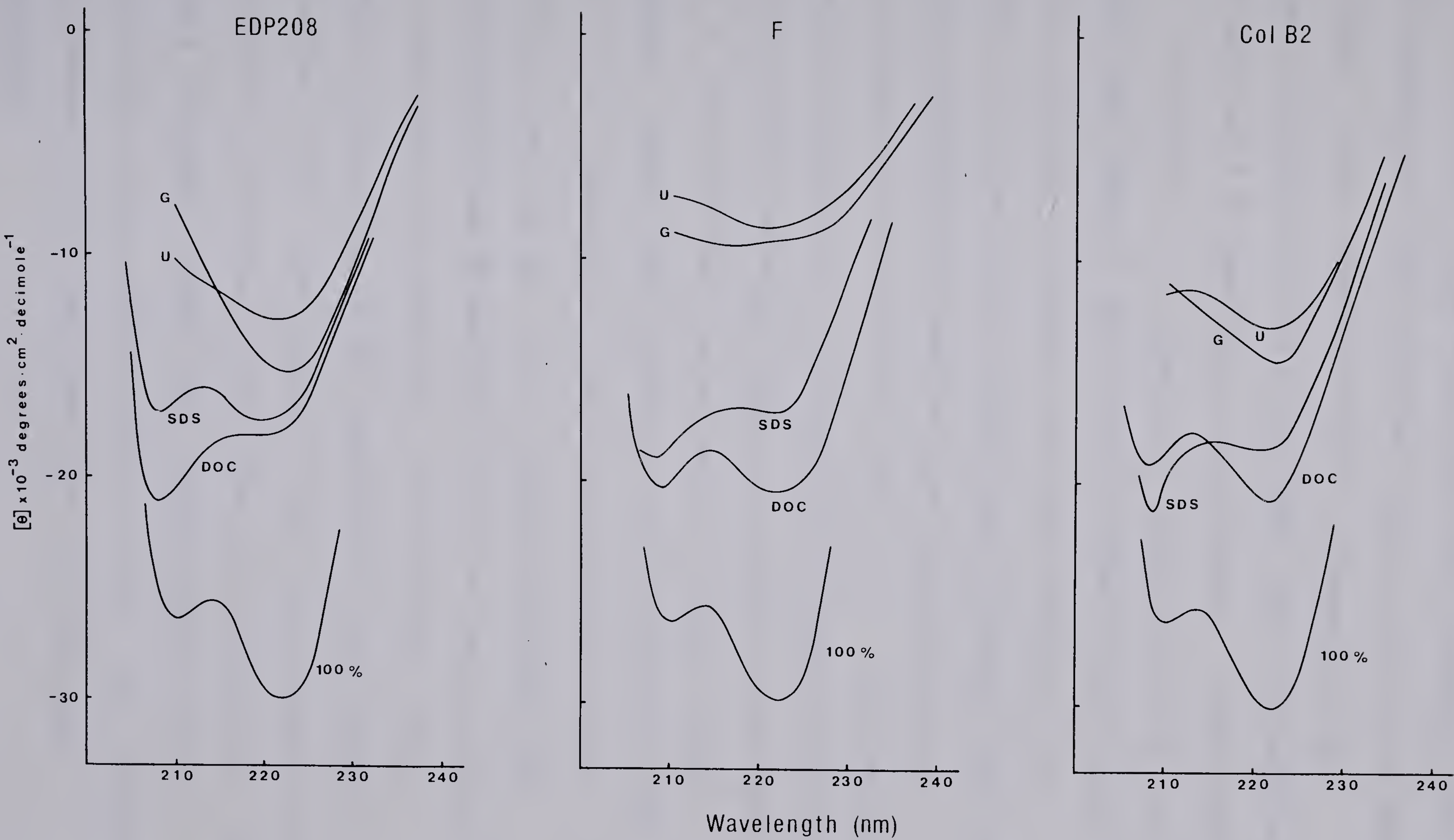
j. Effects of denaturants on EDP208, F, and ColB2 pili

Circular dichroism spectra of intact pili were measured in sodium phosphate buffer (pH 7.2 $\pm$ 0.1) in the presence of either 10 mM deoxycholate, 1.0% SDS, 8 M urea, or 4 M guanidine hydrochloride. Figure 18 shows molar ellipticities for these pili in the wavelength range from 205-235 nm with the bottom curve in each profile being representative of a protein which is 100% α-helical (Chen et al., 1972).

From spectra obtained, the apparent α-helical content of the three types of pilin was calculated using the method of Chen et al. (1972) (see Appendix A), and for pili suspended in 10 mM deoxycholate, the values were in the range of 60-70%. This relatively high value is in agreement with that reported for F pili by Date et al. (1977). It is thought to represent the amount of α-helical structure in native pilin because F pili suspended in 10 mM deoxycholate are morphologically identical to native pili (suspended in buffer alone) and retain 100% of their ability to absorb MS2 phage after removal of the detergent (Date et al., 1977).

Circular dichroism spectra of F and ColB2 pili in buffer alone were not obtained because under these conditions the pili aggregate into large bundles which cause significant light scattering. This, however, was not the case with EDP208 pili so a circular dichroism spectrum for EDP208 pili, suspended in buffer, was obtained.

Figure 18. Circular dichroism spectra of EDP208, F, and ColB2 pilin in the presence of 10 mM deoxycholate (DOC), 1.0% SDS, 8 M urea (U), or 4 M guanidine hydrochloride (G). The pili were suspended in each denaturant in 0.1 M sodium phosphate buffer (pH 7.2 ± 0.1), and spectra were recorded over the wavelength range from 205 to 240 nm. The molar ellipticity was calculated as described in Materials and Methods and is in units of degrees$\cdot$cm$^2\cdot$decimole^{-1}. Protein concentration was determined by amino acid analysis.


EDP208
F
Col B2
[θ] x10^-3 degrees·cm^2·decimole^-1
0
-10
-20
-30
G
U
SDS
DOC
100 %
210
220
230
240
Wavelength (nm)

This spectrum was identical to that of EDP208 pili in 10 mM deoxycholate, thus supporting the contention that pili in the presence of this detergent are in the native state.

Figure 18 also demonstrates the changes in the secondary structure of the pilins when they are exposed to 8 M urea or 4 M guanidine hydrochloride. These agents cause a decrease in the apparent α-helicity in all of the proteins (see Table 7) and it would appear that F pilin is the most sensitive. Despite the noted changes in the secondary structure of all three types of pilin, urea and guanidine hydrochloride seem to exert less effect on subunit-subunit interactions of these pili. When examined by electron microscopy after drying the samples onto the microscope grids and after washing away the denaturants, EDP208, F, and ColB2 pili were found to retain normal morphology. Under these conditions it is unlikely that the pili could reassemble from dissociated subunits. Furthermore, Date et al. (1977) found no evidence for pilus disaggregation by gel filtration in the presence of urea or guanidine hydrochloride. On the other hand, treatment of pili with 1% SDS resulted in the complete dissociation of all three types of pili into fragments which were too small to be seen in the electron microscope. However, this treatment caused only slight alterations in the α-helicity of the three types of pilin. Gel filtration in the presence of SDS indicated that the pili were disaggregated.

Table 7

Apparent α-helical Structure in EDP208, F, and ColB2 Pilin in the Presence of Denaturants

Type of pili	Percent α-helical Structure[a] DOC	SDS	8 M Urea	4 M Guan HCl
EDP208	69 ± 7[b]	55 ± 6	31 ± 3	41 ± 4
F	72 ± 11	57 ± 9	28 ± 4	33 ± 5
ColB2	67 ± 7	62 ± 6	44 ± 4	49 ± 5

a. Calculated using the molar ellipticities at 215, 220, and 225 nm from figure 18 and the equation derived in Appendix A.

b. Standard error of the mean.

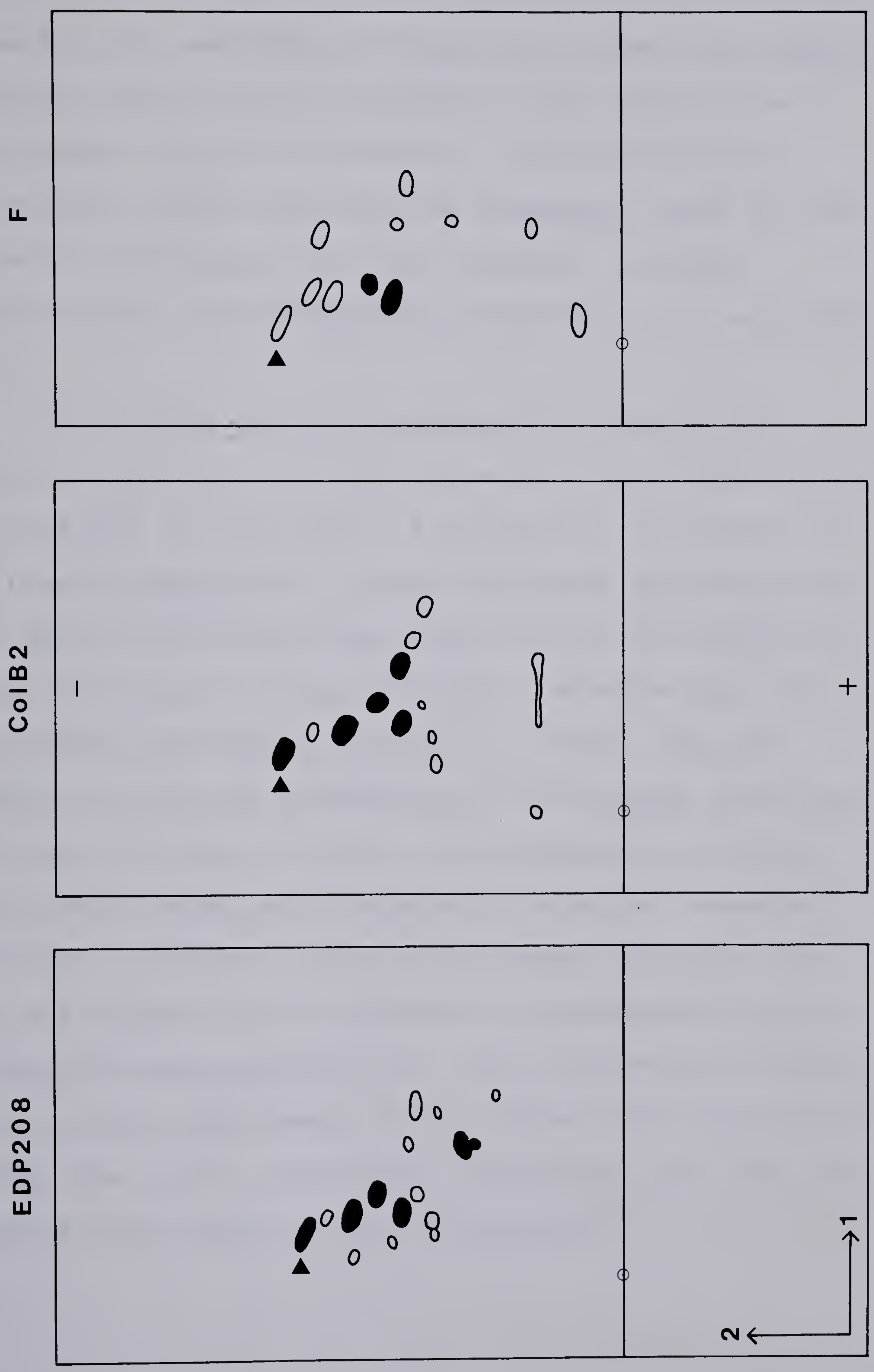
k. Tryptic peptide maps of EDP208, F, and ColB2 pili

When formic acid-denatured EDP208, F, and ColB2 pilin were digested with trypsin as described in Materials and Methods, the majority of the protein remained insoluble. However, two-dimensional chromatography-electrophoresis and amino acid analysis of the supernatant solutions revealed the presence of pili-derived peptides and free amino acids. As shown in figure 19, the EDP208 and ColB2 digests contained five major peptides. The F digest contained only two major peptides, possibly due to the low concentration of peptides in the F digestion mixture. However, two of the F spots co-migrated with two similar spots on the EDP208 and ColB2 maps and the general pattern of all the F spots (major and minor) resembled that of the other pilus types. As indicated in figure 19, one of the spots on each map co-migrated with free lysine. This suggested that the three types of pilin may contain at least one cluster of two or more lysine residues. Minor or faint spots were observed on all of the maps (estimated to represent about 0.5 nmole or less). These were not present in the supernatant solutions before trypsin digestion, and control trypsin digests, to which no pilin was added, produced no spots whatsoever.

3. SUMMARY

The preceding investigations were carried out to elucidate and compare some of the chemical and physical properties of three types of conjugative pili: EDP208,

Figure 19. Two-dimensional chromatography-electrophoresis of soluble tryptic peptides derived from EDP208, F, and ColB2 pilin. Peptides generated from 5 nmole of protein were applied to the origin of microcrystalline cellulose plastic sheets (10 cm x 20 cm) and chromatography was performed in the first dimension using n-butanol-pyridine-water-acetic acid (5:4:4:1 v/v). Electrophoresis was performed in the second dimension using 8% formic-2% acetic acid (pH 2.1). The peptides were stained with the cadmium-ninhydrin reagent. The darkest spots are shaded. The unshaded spots were only faintly visible and probably represent 0.5 nmole or less of peptide. The position of free lysine is indicated by the darts.


F
ColB2
EDP208
−
+
1
2

F, and ColB2. F and ColB2 pili, which serve as attachment sites for the same kinds of F-specific phages (N.S. Willetts, personal communication, see Table 2) were found to be serologically similar structures. On the other hand, EDP208 pili, which only serve as attachment sites for the F-specific DNA-phage, M13 (N.S. Willetts, personal communication) are serologically distinct from F and ColB2 pili.

All of these pili were comprised of one type of protein subunit (pilin) whose molecular weight was estimated to be 10,000, in the case of F and EDP208, and 12,000, in the case of ColB2 pilin. F and ColB2 have basically the same amino acid compositions, whereas that of EDP208 was found to be significantly different. Nevertheless, the three types have many properties in common. They all apparently have both phosphate and carbohydrate associated with them, and they all have a high proportion of hydrophobic amino acids and considerable α-helical secondary structure. Moreover, electron microscopy indicated that they all appeared to be resistant to disassembly by urea or guanidine hydrochloride, but will disaggregate in SDS. However, these experiments did not demonstrate conclusively whether some or all of the pili disaggregate and then reassemble after removal of the denaturants.

CHAPTER V

CHARACTERIZATION OF EDP208- AND ColB2-ASSOCIATED CARBOHYDRATE AND PHOSPHATE

1. INTRODUCTION

As indicated in the previous chapter, both carbohydrate and phosphate were found in association with purified EDP208 and ColB2 pili. Therefore, the following investigations were carried out to determine the nature of these moieties and whether they were covalently linked to the protein.

2. RESULTS AND DISCUSSION

a. Purification of EDP208 and ColB2 pilin monomers

After mild acid hydrolysis as described in Materials and Methods, the pili-associated sugars were identified either by thin layer or gas chromatography. These procedures revealed the presence of at least four sugars associated with each type of pili: glucose, galactose, and an unidentified pentose and dideoxyhexose.

To determine whether these represented pili contaminants, EDP208 and ColB2 pili were dissociated into monomers by dissolving them in 1% SDS. This was followed by passage through Sephadex G-200 gel filtration columns. Figure 20 shows typical elution profiles obtained from these columns and demonstates that the material obtained from pure EDP208 or ColB2 pili was resolved into three peaks. In each case, amino acid analysis and SDS polyacrylamide gel electrophoresis demonstrated that the fractions in the

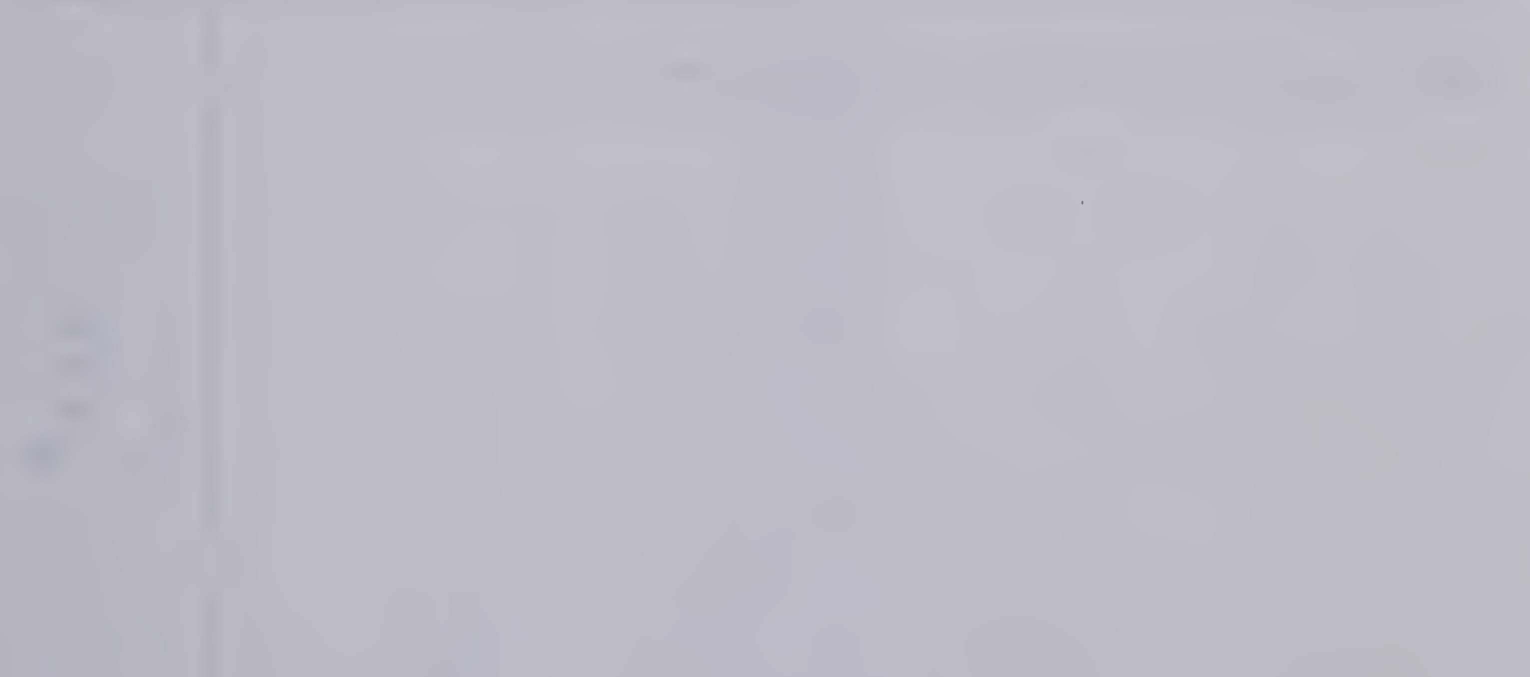
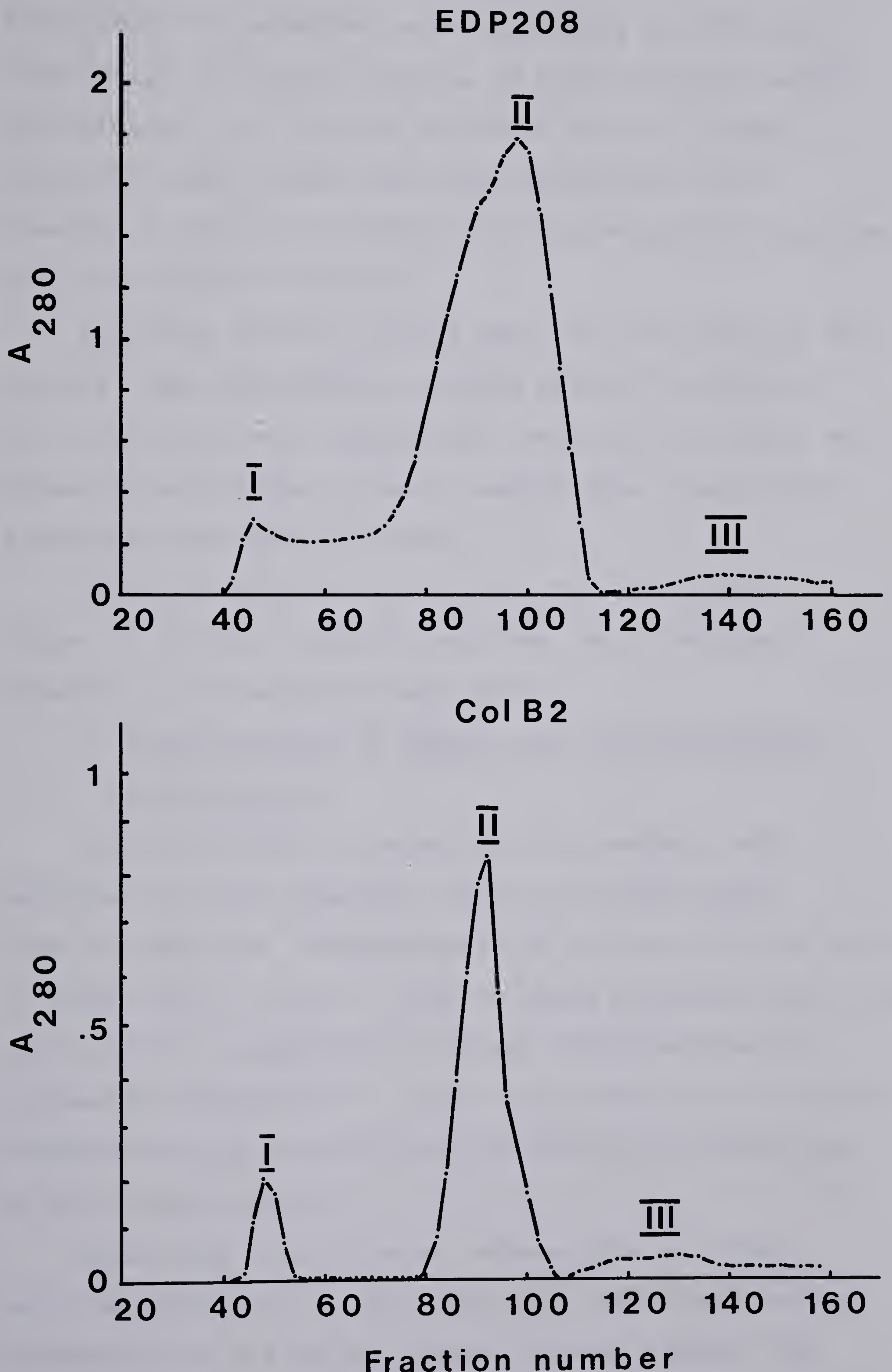


Figure 20. Sephadex G-200 gel filtration chromatography of EDP208 and ColB2 pili. The pili were dissociated into monomers in 1.0% SDS and eluted from the column using 0.1 M Tris-HCl (pH 8.3), 1 mM EDTA, and 1.0% SDS. One ml fractions were collected at a flow rate of 20 ml per hour. The column dimensions were 2.5 cm x 39 cm.


EDP208
A280
2
1
0
I
II
III
20
40
60
80
100
120
140
160
Col B2
A280
1
.5
0
I
II
III
20
40
60
80
100
120
140
160
Fraction number

first peak (I) contained large aggregates of EDP208 or ColB2 pilin plus minute amounts of high molecular weight contaminants. In the case of EDP208 and to a lesser extent of ColB2, these large aggregates were often observed at the top of SDS polyacrylamide gels of purified pili (see figure 13 and 15).

Fractions from the second peak (II) from each of the columns, contained EDP208 or ColB2 pilin. Furthermore, if this material was lyophilized, redissolved in SDS, and passed through G-200 columns a second time, large pilin aggregates were again observed.

Fractions from the third peak (III) contained traces of protein, probably peptides, which were not detected on SDS polyacrylamide gels.

b. Identification of EDP208- and ColB2-associated carbohydrates

Acid hydrolysis or methanolysis procedures were employed to remove possible covalently bound sugars from the proteins. Methanolysis was performed by the method of Clamp, *et al.* (1971). Both of these procedures were first evaluated for carbohydrate recovery using ovalbumin as a standard glycoprotein. Better than 80% of the ovalbumin carbohydrate was removed from the protein and identified by gas chromatography.

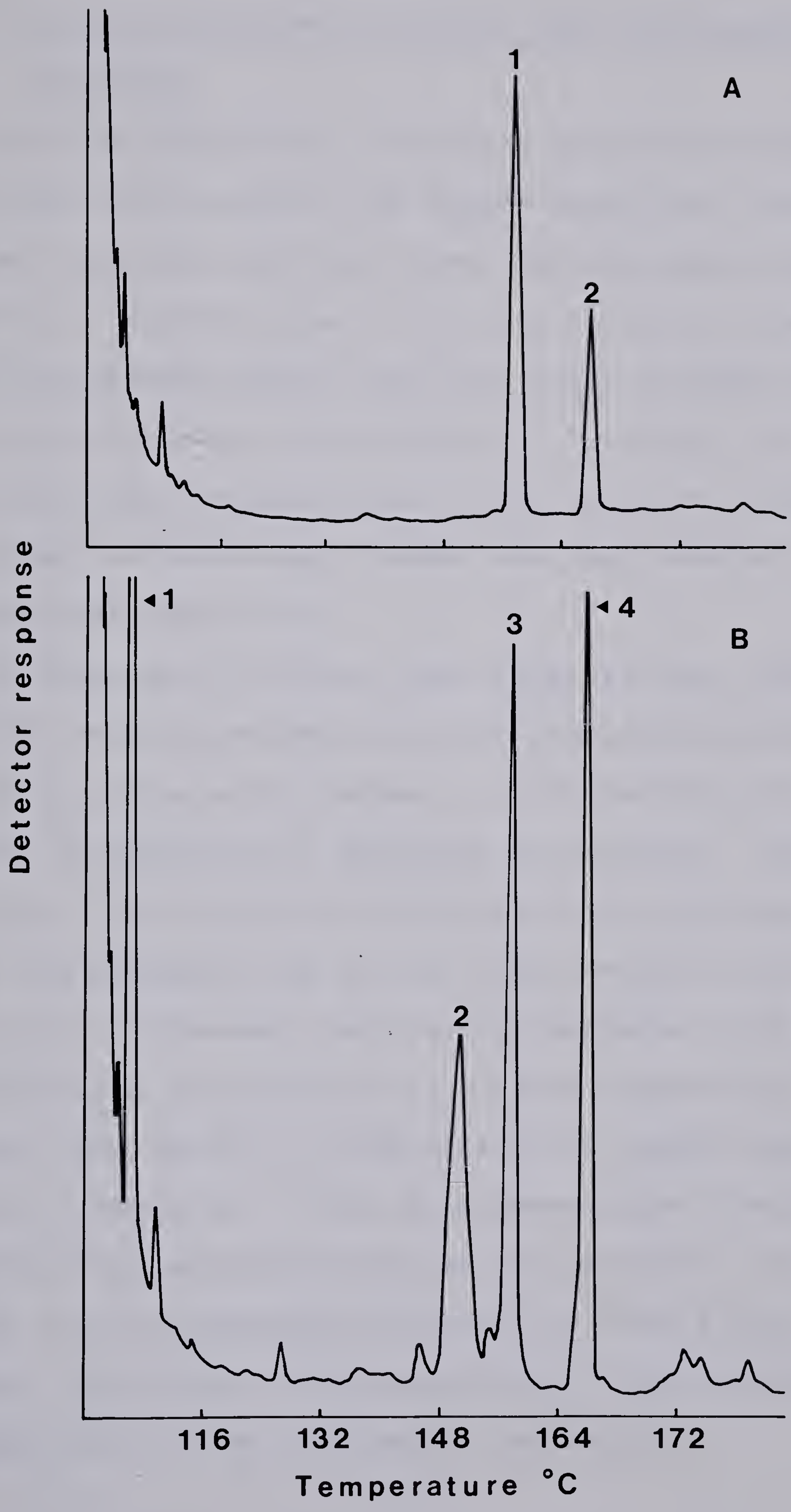
After acid hydrolysis or methanolysis of column-purified EDP208 pilin, thin layer and gas chromatography indicated that all of the sugars had been removed from

the protein. Furthermore, the four sugars were detected in the peak III fractions. It was concluded that EDP208 pilin does not contain covalently bound carbohydrate. The sugars which were removed by SDS column chromatography probably represent contamination from cell surface moieties (such as lipopolysaccharide), since none of them were present in stoichiometric amounts with the protein.

When the same procedures were employed on purified ColB2 pilin, glucose was detected (see figure 21). In figure 21a, standard glucose was resolved into both the α and the β anomers (peaks 1 and 2). This has been observed by others (Clamp et al., 1971; Reading et al., 1978). The same two peaks (3 and 4) were detected on a gas chromatogram of the ColB2 sugars (figure 21b). Furthermore, quantitation of the glucose revealed one glucose per pilin monomer. The first peak (1) in figure 21b, was probably due to a derivitized amino acid, since standard amino acids were detected in this region. The second peak (2) may have been due to an unidentified sugar or to incomplete substitution of the glucose (Reading et al., 1978). If the latter is true then ColB2 pilin may possess more than one glucose per pilin subunit.

covalently bound, purified pilin was precipitated by acetone or extracted with chloroform-methanol. Neither of these procedures removed the sugar. Therefore, it was concluded that ColB2 pilin may contain at least one covalently bound glucose, but it was also possible that it may be bound by some interaction which was not affected

Figure 21. Gas chromatograms of standard glucose (A) and ColB2-associated sugars (B) removed by mild acid hydrolysis. The dried samples were trimethylsilylated with Trisil reagent (Pierce Chemical Co.) according to the manufacturer's direstions, and the derivatized sugars were subjected to gas chromatography on a gas chromatograph equipped with programmable temperature control and a flame ionization detector. The sugars were resolved by increasing the temperature from $100^{o}C$ to $230^{o}C$ at a rate of $8^{o}C$ per minute. The two glucose peaks represent the α and β anomers which are both generated by acid hydrolysis.


1
A
2
◂1
3
◂4
B
2
Detector response
116
132
148
164
172
Temperature °C

by SDS or acetone precipitation.

c. Characterization of EDP208- and ColB2-associated phosphate

Although the SDS gel filtration procedure resulted in the removal of most of the sugars from pilin, this treatment did not affect the level of pilin-associated phosphate. Determination of the total inorganic phosphate in column-purified EDP208 and ColB2 pilin revealed the presence of phosphate (see Table 8). Therefore, the pilin-associated phosphate was either covalently bound to the protein or associated through some non-covalent SDS-resistant interaction.

To distinguish between these possibilities, EDP208 and ColB2 column-purified pilin was precipitated from the SDS solutions with acetone or extracted with chloroform-methanol as described in Materials and Methods. Phosphate analysis of the acetone-precipitated pilin indicated that 97% of the phosphate was removed from EDP208 and 86%, from ColB2 pilin. Moreover, the missing phosphate could be quantitatively recovered in the acetone supernatant solutions (see Table 8). The failure of acetone precipitation to remove all of the ColB2-associated phosphate as efficiently as EDP208 phosphate may reflect a tighter binding of the phosphorylated moiety to ColB2 pilin. However, the existence of one covalently bound phosphate in ColB2 pilin was an alternative possibility.

Table 8

Fate of Pili-associated Phosphate After Acetone Precipitation

Fraction	Type of pilin	nmole of pilin[a]	nmole of phosphate[b]	Phosphate per pilin
G-200 purified	EDP208	112	325	2.9
peak II pilin	ColB2	25.0	75.0	3.0
Acetone[c]	EDP208	97.0	< 8.0	< 0.08
Precipitate	ColB2	20.8	8.1	0.39
Acetone Supernatant	EDP208	< 8.0	270	-
Solution	ColB2	< 8.0	53	-

a. Determined by amino acid analysis of samples hydrolysed in 6 N HCl for 24-48 hours.

b. Determined by the method of Chen et al. (1956) for inorganic phosphate after hydrolysing samples for 72 hours in 6 N HCl. The detection limit of the assay was 5 nmoles.

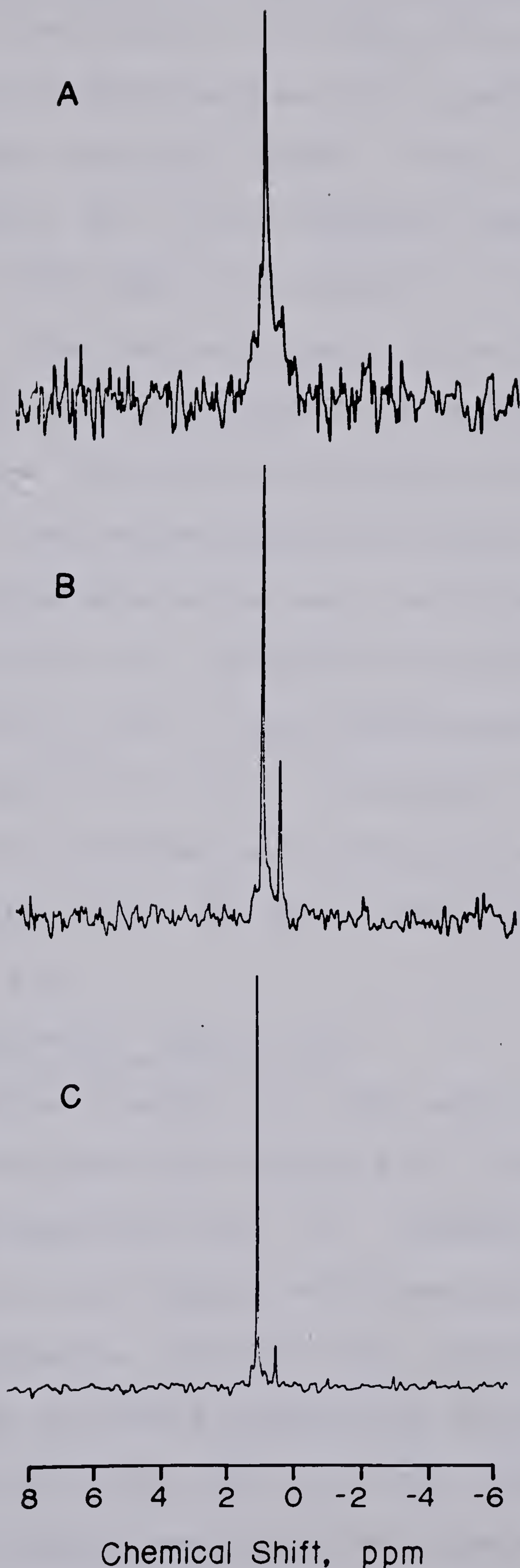
c. The precipitate was collected by centrifugation at 10,000 x g for 20 minutes after the addition of 4 volumes of spectral grade acetone to 1-2 mg/ml solutions of column-purified pilin in 1.0% SDS.

d. ^{31}P NMR of EDP208 and ColB2 pilin in SDS

With a view toward elucidating the nature of the phosphate linkages in EDP208 and ColB2 pilin and to determine whether ColB2 pilin possessed any covalently bound phosphate, column-purified pilins were examined in the presence of SDS by ^{31}P NMR spectroscopy. Figure 22 (panels A and B) shows the ^{31}P NMR spectra of EDP208 and ColB2 pilin, respectively. These are essentially the same and display one rather broad downfield resonance at about 1 ppm and a minor resonance at 0.6 ppm. Titration of the samples in the 6-10 pH range did not result in any significant shifts of peak positions. Moreover, the addition of approximately 1 mM manganese ions caused severe, EDTA-reversible, peak broadening. The foregoing observations, as well as results of proton decoupling studies (Vogel and Armstrong, unpublished results), were indicative of a phosphodiester moiety in both proteins rather than a phosphomonoester as usually seen in phosphoproteins (Cozzone and Jardetzky, 1976; Burs *et al.*, 1979; Edmonson and James, 1979).

As shown in figure 22c, the acetone supernatant solution of EDP208 pilin was similar to that of whole unprecipitated EDP208 pilin. This indicated that the phosphodiester bond did not involve a covalent linkage with the protein. Identical results were obtained with the acetone supernatant of ColB2 pilin.

Figure 22. ^{31}P NMR spectra of whole column-purified EDP208 (A) and ColB2 (B) pilin dissolved in 1.0% SDS, and of the acetone supernatant solution of EDP208 (C). The spectra were obtained at a frequency of 109.3 MHz on a Brüker HXS 270 spectrophotometer locked onto the resonance of 20% D_2O in the samples. Chemical shifts were referenced to an 85% phosphoric acid external standard, and downfield shifts were given a positive sign.


A
B
C
8
6
4
2
0
-2
-4
-6
Chemical Shift, ppm

e. Identity of pili-associated phosphate

Column-purified EDP208 and ColB2 pilins were also extracted with chloroform-methanol by a modification of the Bligh and Dyer technique (1959). This procedure removed essentially all of the phosphate from EDP208 and about 80% from ColB2 pilin (see Table 9). Furthermore, silica gel thin layer chromatography of the chloroform-methanol extracts and visualization of the phospholipids with iodine vapors revealed the presence of both phosphatidylglycerol and phosphatidylethanolamine (see figure 23). Phosphatidylethanolamine was also detected on thin layer chromatograms using the cadmium-ninhydrin spray. As indicated in Table 9, all of the EDP208-associated phosphate and about 77% of that associated with ColB2 pilin could be recovered as these two phospholipids. No phosphate was detected in the minor spots seen on the chromatogram in figure 23.

f. ^{32}P-labeling of EDP208 pili

To gain further insight into the nature of the phospholipid associated with EDP208 pili, attempts were made to label EDP208 pili with ^{32}P. However, since liquid cultures do not contain sufficient pili to permit efficient precipitation with PEG 6000, unlabeled pili were added to the cell-free supernatant solutions to facilitate pilus precipitation. As a control, unlabeled pili were also precipitated from cell-free supernatant solutions of ^{32}P-labeled cultures of the plasmidless strain, JC6256.

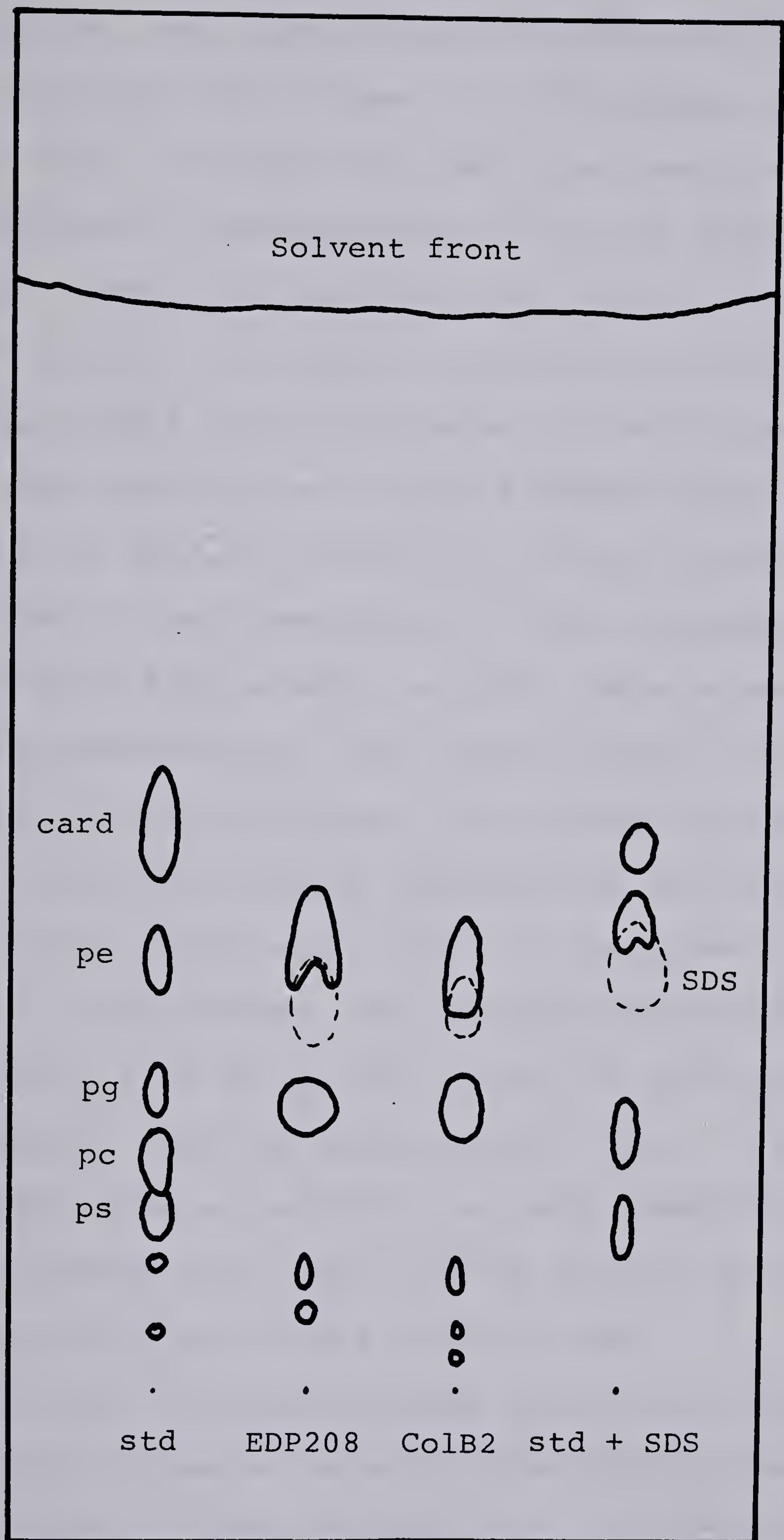
Table 9

Quantitation of Chloroform-methanol Extracted Phospholipids from EDP208 and ColB2 Pilins

Fraction	Type of pilin	nmole of pilin present	nmole of phosphate	Phosphate per pilin
G-200 purified	EDP208	166	473	2.9
peak II pilin	ColB2	161	499	3.1
Chloroform	EDP208	n.d.[a]	340	-
layer	ColB2	n.d.	370	-
Chloroform precipitated	EDP208	135	< 5	< 0.04
pilin pellet	ColB2	131	47	0.60
phosphatidyl-	EDP208	-	220	-
glycerol	ColB2	-	150	-
phosphatidyl-	EDP208	-	120	-
ethanolamine	ColB2	-	135	-

a. none detected.

Figure 23. Thin layer chromatography of chloroform-methanol extracts from EDP208 and ColB2 pili. Standard mixtures included 10 μg each of cardiolipin (card), phosphatidylethanolamine (pe), phosphatidylglycerol (pg), phosphatidylcholine (pc), and phosphatidylserine (ps). A second standard mixture also contained 10 μg of SDS, since this was present in the chloroform-methanol extracts of pilin and affected the mobilities of pilus-associated lipids. The chromatography solvent system was chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5). Phospholipid spots were visualized by iodine vapors or in the case of phosphatidylethanolamine and phosphatidylserine by spraying the chromatograms with the cadmium-ninhydrin reagent and heating at 60°C for 5 minutes. SDS was visualized by charring the chromatograms at 350°C after spraying with 3% potassium chromate in 60% sulfuric acid.


Solvent front
card
pe
pg
pc
ps
SDS
std
EDP208
ColB2
std + SDS

Surprisingly, when these pili were dissolved in SDS and passed through G-200 columns, the ^{32}P elution profiles in each case displayed the same three peaks (see figure 24). Moreover, autoradiography of an SDS polyacrylamide slab gel of the peak fractions (see figure 25) indicated that the ^{32}P in peak I was mostly associated with the high molecular weight pilin aggregates, although some migrated at the same position as monomeric EDP208 pilin. The ^{32}P compounds in peak III moved with the dye marker. The ^{32}P in peak II was associated with the Coomassie blue stained pilin band as well as with a band of material (probably phospholipid) that moved slightly faster than the pilin, but did not stain with Coomassie blue. The latter observation further demonstrated that the phospholipids remain bound to the pilin in the presence of SDS. However, it was evident that the phospholipids which are released into the growth medium are efficiently absorbed to previously purified pilus preparations. It was not determined, however, whether the newly absorbed phospholipids exchange with those already present on the pili or whether they are simply added to them.

When the ^{32}P-labeled EDP208 pilin was precipitated with acetone or extracted with chloroform-methanol, about 75% of the counts were removed into the acetone supernatant solutions. Autoradiography of thin layer chromatograms of these revealed the presence of phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and phos-

Figure 24. Sephadex G-200 column chromatography of ^{32}P-labeled EDP208 pili purified from cell-free supernatant solutions of ED3873 (A) and JC6256 (B). The pili were dissociated in 1.0% SDS then chromatographed on G-200 columns (1 cm x 23 cm) using 0.1 M Tris-HCl (pH 8.3), 1 mM EDTA, and 1.0% SDS. Fractions (0.4 ml) were collected at a flow rate of about 7.5 ml per hour.

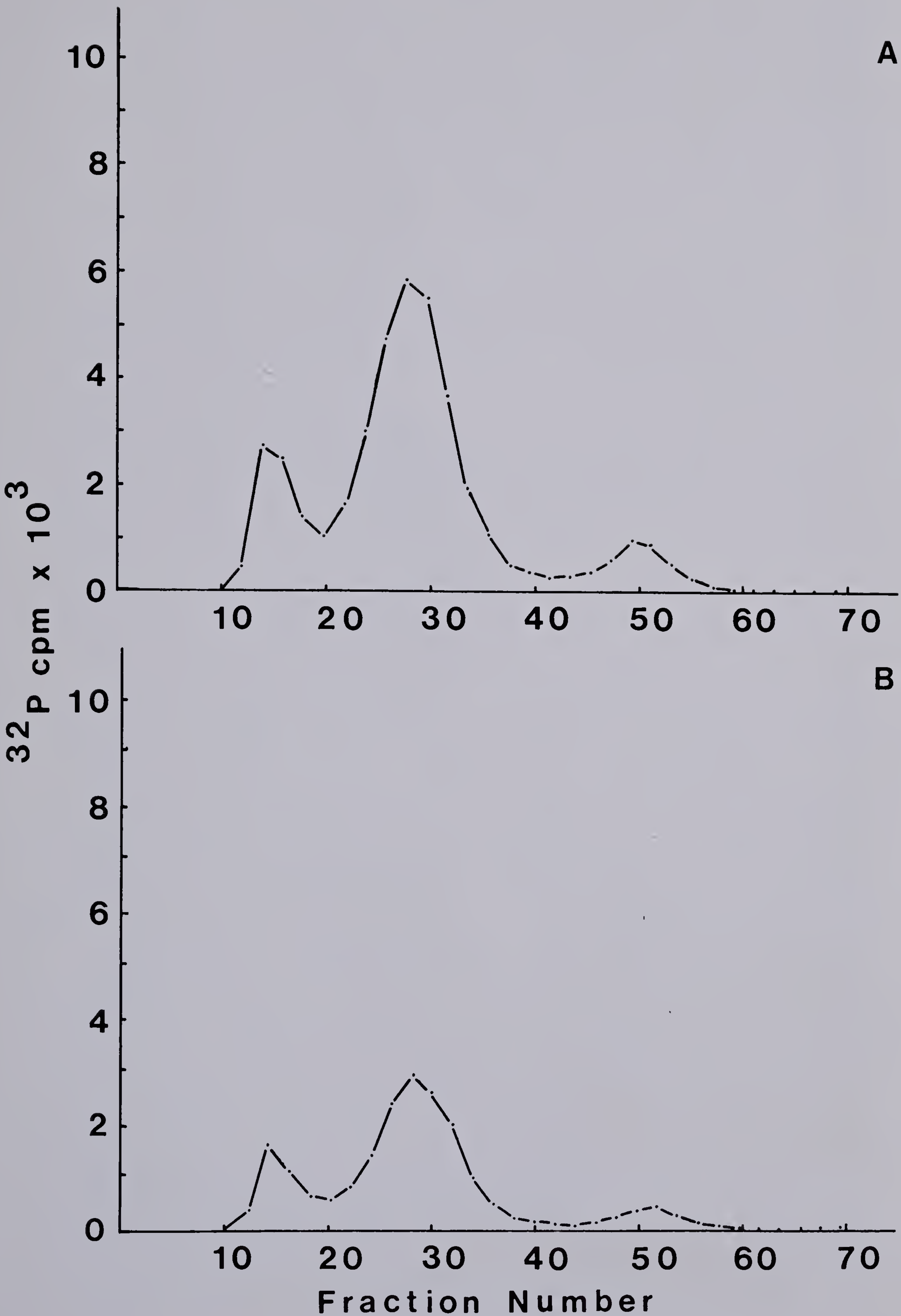
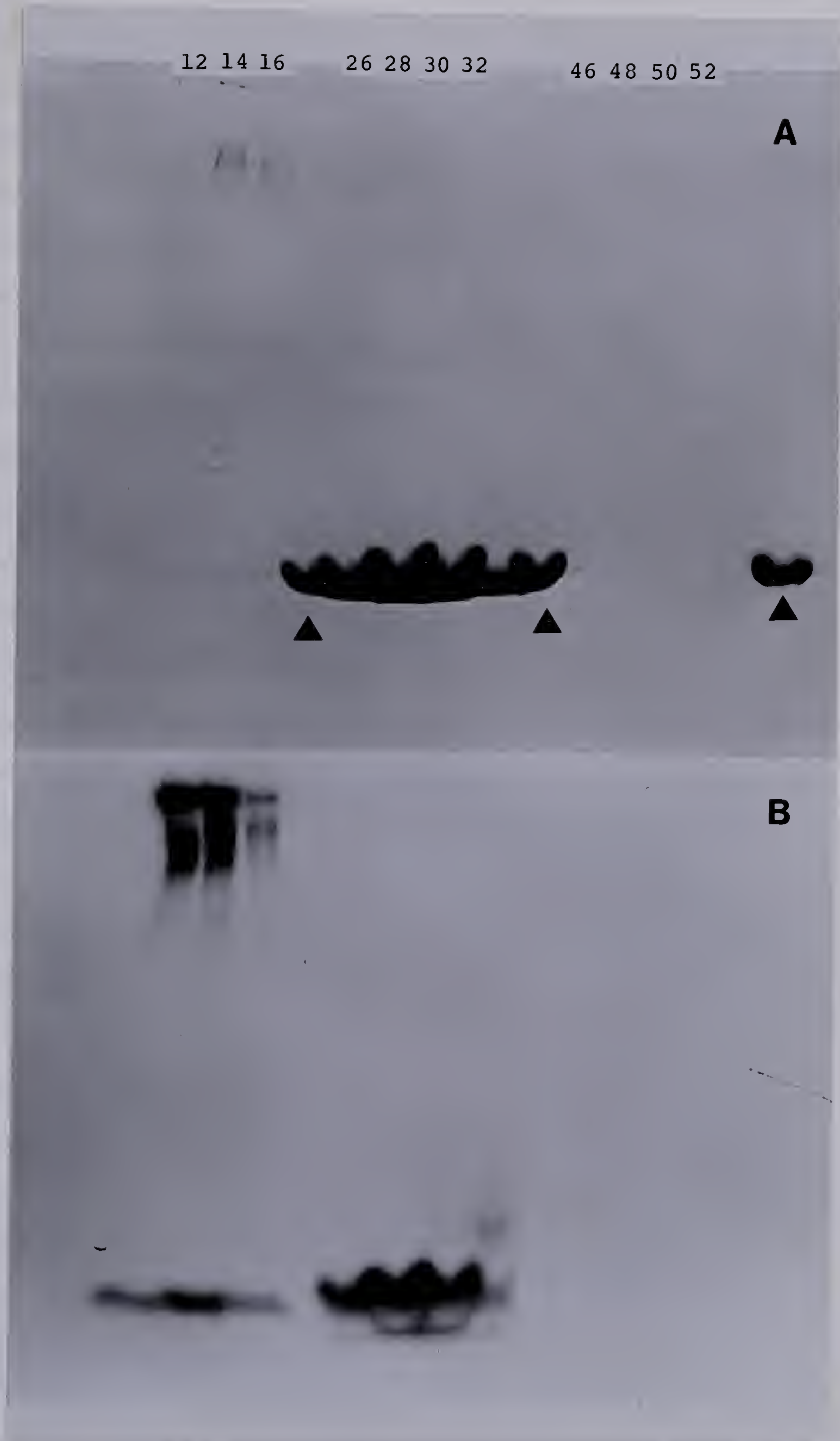

A
B
10
8
6
4
2
0
^{32}P cpm x 10^3
10 20 30 40 50 60 70
Fraction Number

Figure 25. Stained SDS slab gel electropherogram (A) and autoradiogram (B) of ^{32}P-labeled EDP208 pilin from ED3873 cell-free supernatant solutions. Fifty μl of the selected column fractions, indicated by the numbers above each gel slot, were electrophoresed at 50 ma until the bromophenol blue dye marker had migrated approximately 9 cm from the origin. The fraction numbers correspond to the elution profile shown in figure 24a. After electrophoresis, the gel was fixed for 12 hours in a solution containing 40% methanol and 10% acetic acid, then stained by the method of Fairbanks _et al_. (1971). To detect ^{32}P-labeled compounds, the gel was exposed to Kodak X-ray film for 72 hours. Unlabeled EDP208 pilin was also electrophoresed in the positions indicated by the darts (▲).


12 14 16
26 28 30 32
46 48 50 52
A
B

phatidylglycerol. Quantitation of the ^{32}P on these chromatograms indicated that 53% of the EDP208-associated phospholipid was phosphatidylglycerol, 30% phosphatidylethanolamine, 4% phosphatidylcholine, and 13% phosphatidylserine. This result was the same regardless of the cell-free supernatant solution from which the pili were obtained. It was therefore concluded that EDP208 readily absorb phospholipids present in the cell-free bacterial growth medium, and that the phospholipids probably represent contaminants of the pili preparations. On the other hand, quantitation of the phospholipids in ED3873 membranes, revealed that 90% of the membrane phospholipid was phosphotidylethanolamine and only about 10% was phosphotidylglycerol. Therefore, it seemed unusual that the major phospholipid associated with EDP208 pilin was phosphatidylglycerol. This suggests that there may be some functional relationship between phosphatidylglycerol and EDP208 pili or that phosphatidylglycerol is preferentially released into the medium.

Since acetone precipitation failed to remove all of the radioactivity from EDP208 pilin, the possibility existed that other phosphate moieties, perhaps nucleic acids or nucleotides, were also present in the preparations. Accordingly, column-purified pilin, obtained from either of the labeled cell-free supernatant solutions, was extracted with formic acid. Ascending chromatography of the formic acid supernatant solutions on DEAE-cellulose

revealed the presence of all four ribonucleotides after autoradiography of the chromatograms. However, quantitation of these by high pressure liquid chromatography indicated that their concentrations were extremely low ($<$ 0.1 mole per 8 mole of pilin). These components were therefore considered to be minor pilus contaminants.

3. SUMMARY

Previous chemical analyses of EDP208 and F pilin indicated the presence of 1-2 mole of phosphate and about 1% by weight of carbohydrate (Brinton, 1971; Date et al., 1977; Armstrong et al., 1980). However, these investigations failed to determine whether these components were covalently bound to the protein. The experiments described herein indicate that EDP208 pili contain neither covalently bound carbohydrate nor phosphate. Furthermore, the experiments suggest that the carbohydrate and phosphate probably represent contaminants of the pilus preparations.

On the other hand, ColB2 pilin apparently possesses at least one covalently bound glucose molecule and may contain one covalently bound phosphate as well. However, it is also possible that the glucose and phosphate associated with ColB2 pilin are not covalently bound, but extremely tightly associated with the pilin in a manner which makes them resistant to SDS or chloroform-methanol extraction. To determine whether these components are indeed covalently attached to the protein, it will be

necessary to isolate peptides which contain phosphate and glucose.

CHAPTER VI

N-TERMINAL ANALYSIS OF EDP208 AND ColB2 PILIN

1. INTRODUCTION

Preliminary attempts to elucidate the N-terminal amino acid sequence of EDP208 and ColB2 pilin failed due to the presence of a blocking group. The purpose of the following investigation was to elucidate the nature of the blocking group and to determine as much as possible of the N-terminal amino acid sequence of both proteins.

2. RESULTS AND DISCUSSION

a. Isolation of blocked N-terminal peptides from EDP208 and ColB2 pilin

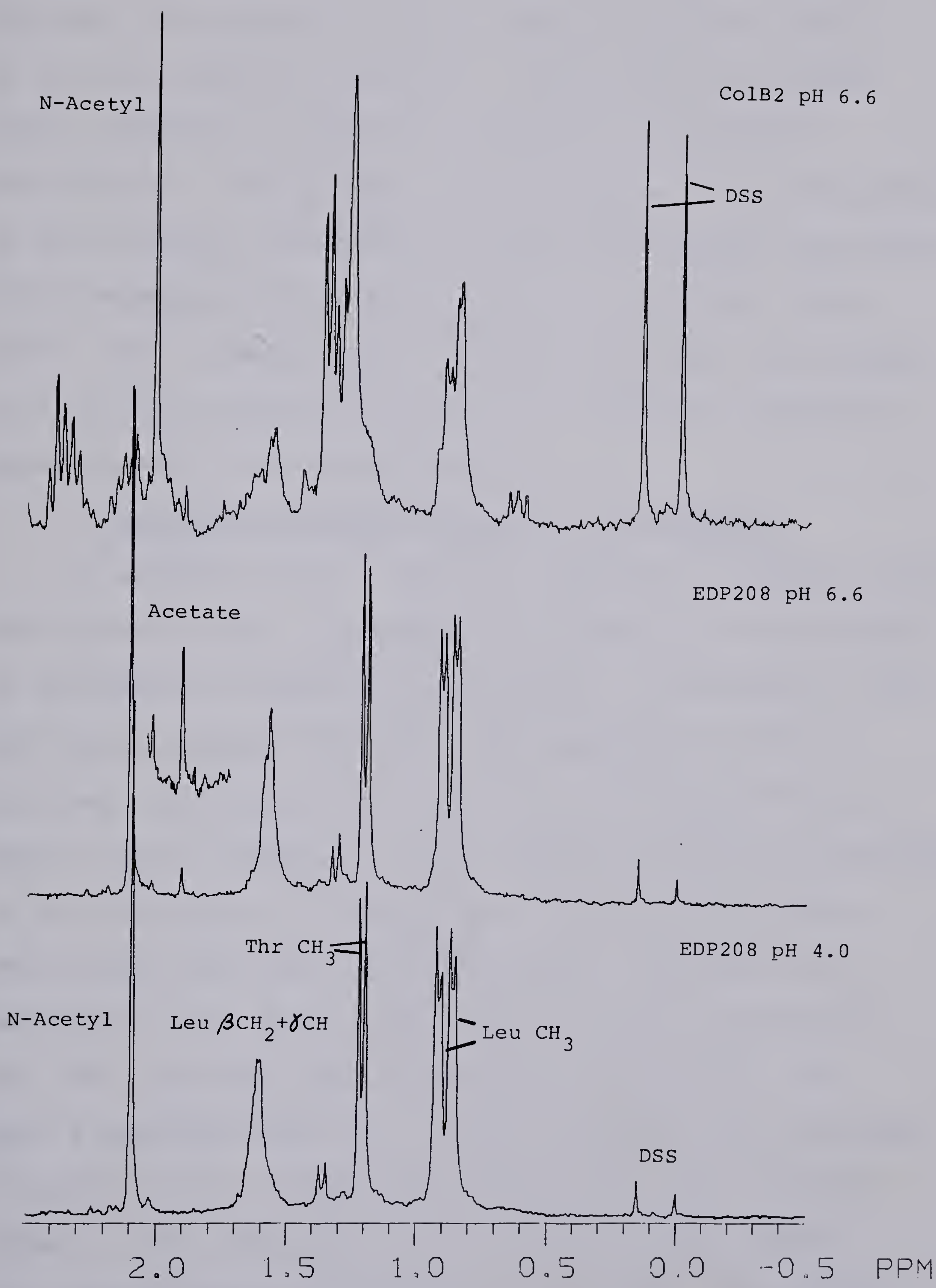
When acetone-precipitated pilin was digested with pronase, two-dimensional chromatography-electrophoresis revealed that soluble peptides were released from the protein. The N-terminal peptides from EDP208 and ColB2 pilin were isolated by passing the soluble pronase-peptides of each protein through a cation exchange column as described in Materials and Methods. Acidic peptides were eluted with water, but in each case the cadmium-ninhydrin spray failed to detect any peptides when unhydrolysed aliquots of the water eluates were electrophoresed on thin layer cellulose sheets (pH 1.8). However, when total acid hydrolysed aliquots of the water eluates were subjected to thin layer electrophoresis, at least two ninhydrin reactive spots were detected for each type

of pilin. Therefore, the water eluates probably contained small peptides which did not contain free amino groups before acid hydrolysis. Amino acid analysis of these revealed the composition of the EDP208 peptide to be Asp, Thr, Leu (1:1:1) and that of the ColB2 peptide to be Glu, Asp, Gly, Leu, Ala (2:1:1:1:1) (see Appendix B). Furthermore, the amino acid analysis indicated the presence of one mole of peptide per mole of each type of pilin. To show conclusively that these peptides had blocked N-terminals, 20 nmole of unhydrolysed peptide from each were subjected to thin layer electrophoresis. The cadmium-ninhydrin spray, which was able to detect as little as 0.5 nmole of free amino group, failed to reveal either of the N-terminal peptides, although trace spots ($<$1.0 nmole) of other amino acids were detected in the heavily overloaded electropherograms.

b. Identification of blocking group

Identity of the blocking group was revealed by ^{1}H NMR (see figure 26). As indicated in the figure, resonance peaks at 2.1 ppm (EDP208) and 2.0 ppm (ColB2) probably represent N-acetyl groups. This assignment was made based on the following observations. (1) The resonances were at similar positions to those of the acetyl moiety on standard N-acetyl aspartic acid or N-acetyl threonine. (2) The resonances were located at different positions than that of the free acetate (1.9 ppm). This was included in the samples as an internal standard. (3) Titration of

Figure 26. 270 MHz ^1H NMR spectra of ColB2 and EDP208 N-terminal peptides. To obtain the spectra, 160 nmole of ColB2 and 500 nmole of EDP208 peptide were dissolved in 99.8% D_2O (Bio Rad) containing 5 mM imidazole, as a buffer, and 0.05 mM DSS (sodium 2,2-dimethyl-2-silapentane-5-sufonate) as an internal standard. Spectra for the EDP208 tripeptide were obtained at pH 6.6 and pH 4.0.



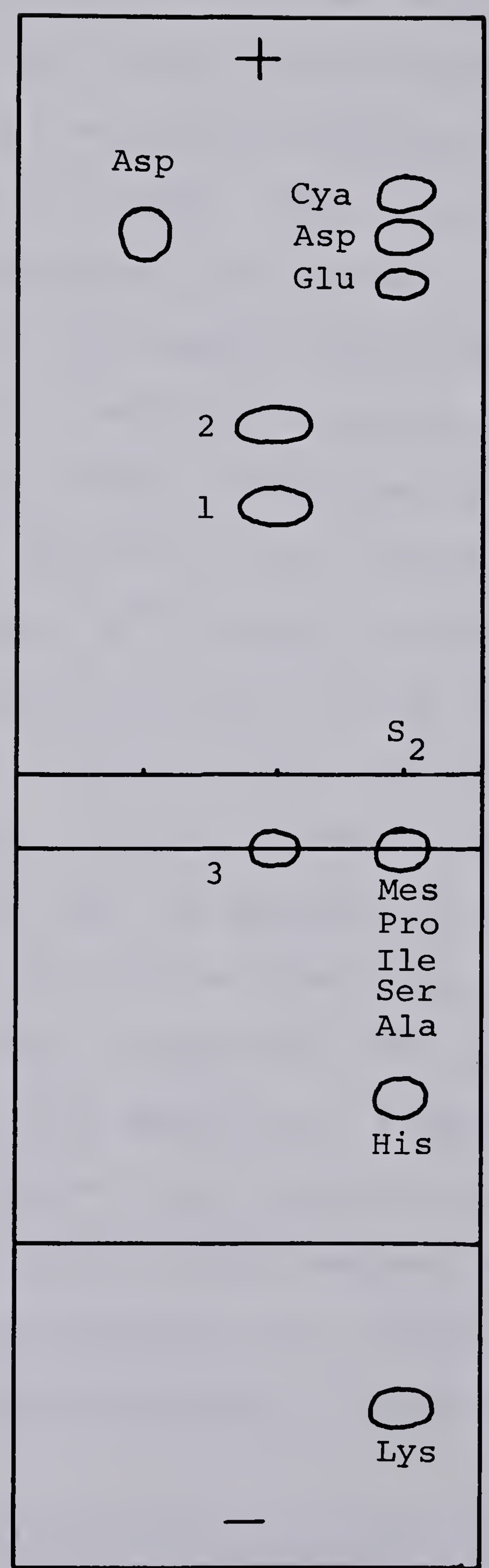
N-Acetyl
ColB2 pH 6.6
DSS
Acetate
EDP208 pH 6.6
Thr CH3
EDP208 pH 4.0
N-Acetyl
Leu βCH2+γCH
Leu CH3
DSS
2.0
1.5
1.0
0.5
0.0
-0.5
PPM

the samples from pH 6.6 to pH 2.0 failed to cause the N-acetyl resonances to shift position significantly, whereas pH titration caused a severe downfield shift of the free acetate resonance. Both EDP208 and ColB2 pilin, therefore, appeared to possess N-acetylated N-terminals. Furthermore, the difference in the positions of the N-acetyl resonances indicated that EDP208 and ColB2 pilin probably do not share the same N-terminal amino acid. The ^{1}H NMR spectra of EDP208 pilin was also useful in that they demonstrated that the tripeptide contained aspartic acid and not asparagine.

c. Sequence of EDP208 N-terminal tripeptide

To sequence the N-terminal tripeptide of EDP208 pilin, the N-acetyl group was removed by limited acid hydrolysis as described in Materials and Methods. Separation of the hydrolysis products by paper electrophoresis (pH 6.5) produced three spots (see figure 27). The mobilities of spots 1 and 2 relative to free aspartic acid were calculated to be 0.53 and 0.67, respectively. When the peptides were eluted from the electropherogram, the amino acid analyses of the three indicated that spot 1 contained Asp, Thr, and Leu, spot 2 contained Asp and Thr, and spot 3 contained only Leu. Using the amino acid analyses of spots 1 and 2, theoretical mobilities were assigned (spot 1, 0.53; and spot 2, 0.7) which agreed closely with their observed mobilities (see above). N-terminal analysis of peptide 1 by the dansylation procedure

Figure 27. Scale drawing of paper electropherogram of partial acid hydrolysis products of the EDP208 N-terminal tripeptide. Twenty to thirty nmoles of peptide were applied to the origin and electrophoresis was carried out using 10% pyridine, 0.3% acetic acid (pH 6.5). The conditions were 3000 V for 45 minutes. The spots were visualized with cadmium-ninhydrin spray. The mobilities of spots 1 and 2 were calculated relative to the distance between the center of the neutral spot and the top of the aspartic acid spot. Standard (S_2) contained Pro, Ile, Ser, Ala, His, Lys, Glu, Asp, methionine sulfone (Mes) and cysteic acid (Cya).


+
Asp
Cya
Asp
Glu
2
1
S_2
3
Mes
Pro
Ile
Ser
Ala
His
Lys
—

indicated that the N-terminal of the tripeptide was threonine. Therefore, since the dipeptide (spot 2) contained Thr and Asp, the entire sequence of the N-terminal tripeptide of EDP208 pilin was deduced to be acetyl-NH-Thr-Asp-Leu.

The sequence of the EDP208 N-terminal tripeptide was also determined by ^{1}H NMR. This involved the use of Yb^{3+} (Ytterbium) as a paramagnetic shift probe. Ions such as Yb^{3+} markedly affect the magnetic environment of nuclei within their vicinity producing shifts in line positions and altered relaxation rates. The effect on relaxation rate decreases as a function of the distance between the proton and Yb^{3+}. Since Yb^{3+} binds to carboxyl groups, titration of the tripeptide with this ion allowed the distance between the carboxyl groups and characteristic protons to be determined. The data (Edwards, personal communication) suggested that the sequence of the tripeptide was the same as that determined chemically.

When the N-terminal hexapeptide from ColB2 was subjected to limited acid hydrolysis, a hexapeptide with an unblocked N-terminal was not detected in the hydrolysate. Unfortunately, the ColB2 hexapeptide was not available in sufficient quantity for further analysis. Therefore, the N-terminal sequence of ColB2 was not determined.

d. Isolation of N-terminal tripeptide from trypsin digests of EDP208 pilin

Since the amino acid composition of the EDP208

soluble pronase peptides resembled that of the soluble tryptic peptides (see Table 10), it seemed likely that a longer N-terminal peptide could be isolated from a trypsin digest of EDP208 pilin. Accordingly, 500 nmole of EDP208 pilin was trypsin-digested and the soluble portion of the digest was passed through a Bio Gel P2 gel filtration column. As indicated in figure 28, six peaks were resolved, the major one being peak 1. The peak 1 fractions were, therefore, pooled and lyophilized for analysis. Two-dimensional thin layer chromatography-electrophoresis revealed the presence of three peptides which co-migrated with three of the spots which appeared on the tryptic maps of EDP208 pilin (see figure 29). To determine if one of these peptides possessed a blocked N-terminal, an aliquot of the P2-1 pooled fractions was further digested with pronase and the digest was passed through a cation exchange column, as described previously. The amino acid analysis of the water eluate showed the presence of a tripeptide whose composition was Asp, Thr, Leu (1:1:1). Partial acid hydrolysis and paper electrophoresis of this tripeptide showed it to be the same as that released from whole pilin by pronase digestion. The amino acid analysis also showed that 500 nmole of tripeptide was released. Therefore, trypsin digestion also quantitatively removes the N-terminal of EDP208 pilin. The three trypsin peptides were not purified and characterized further during the course of this research.

Table 10

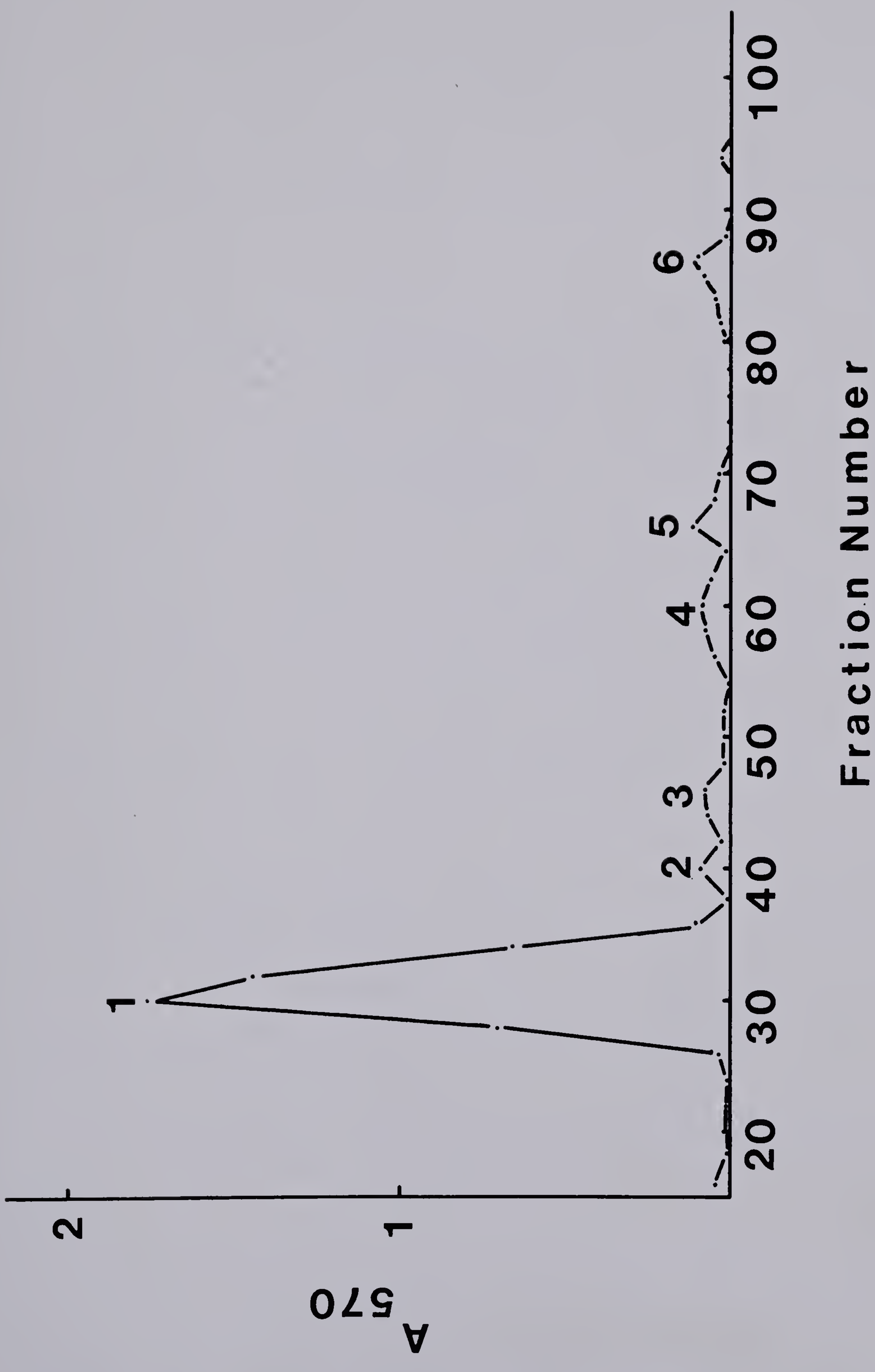
Amino Acid Composition of Soluble Pronase and Tryptic Peptides Derived from EDP208 Pilin

Amino Acid	Percent of total[a]	
	Pronase-soluble	Trypsin-soluble
Lys	13.2	14.1
His	0	0
Arg	0	0
Asp	20.6	21.4
Thr	12.8	8.4
Ser	2.3	3.4
Glu	1.6	3.0
Gly	12.5	16.0
Ala	11.5	8.7
½Cys	n.d.[b]	n.d.
Val	6.0	7.3
Met	0.8	0.8
Ile	2.2	1.5
Leu	18.5	15.5
Tyr	< 0.5	< 0.5
Phe	4.3	< 0.5

a. The amount of each amino acid as a percentage of the total amount of amino acids present.

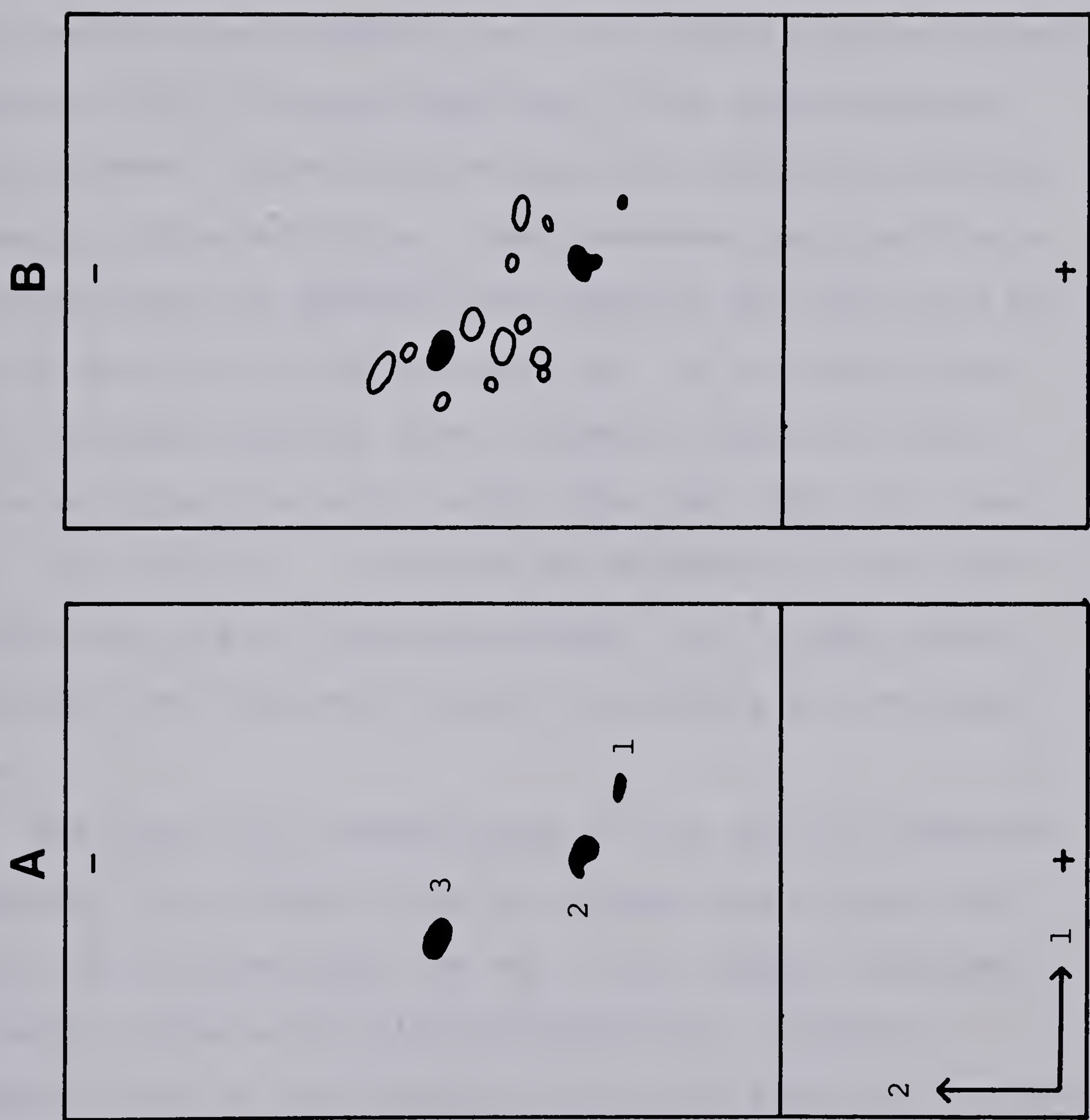
b. not determined.

Figure 28. Gel filtration elution profile of EDP208 soluble tryptic peptides. The column (1 cm x 80 cm) was packed with Bio Gel P2 (exclusion limit 1800) in 0.1 M ammonium bicarbonate pH 8.0. The peptides were dissolved in 0.5 ml ammonium bicarbonate and eluted with the same at a flow rate of approximately 8 ml per hour. The volume of each fraction was approximately 0.5 ml. The peptides were located by reacting an aliquot of every second fraction with ninhydrin, as described in Materials and Methods, and reading their absorbances at 570 nm.



A_{570}
1
2
Fraction Number
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6

Figure 29. Thin layer two-dimensional chromatography-electrophoresis of EDP208 pilin soluble tryptic peptides from the peak 1 fractions of a Bio Gel P2 gel filtration column (A). Approximately 5 nmole of peptides were applied to the origin of a microcrystalline sheet (10 cm x 20 cm) and ascending chromatography was performed in the first dimension using n-butanol-pyridine-water-acetic acid (5:4:4:1 v/v). Electrophoresis was in the second dimension for 45 minutes at 500 V using 8% formic-2% acetic acid (pH 2.1). The peptides were stained with the cadmium-ninhydrin reagent. For comparison purposes, the trypsin peptide map of all the soluble peptides is also shown (B). The shaded spots in B co-migrate with those in A.


A
B
−
+
1
2
3

3. SUMMARY

In chapter III of this thesis, it was shown that the N-terminal of EDP208, F, and ColB2 pilin were blocked and therefore failed to react with dansyl chloride. N-terminal peptides have now been isolated from the soluble fraction of pronase digested EDP208 and ColB2 pilin and the blocking group on each has been identified as an acetyl moiety. This, however, appears to be the only similarity between these two types of pilin. The N-terminal peptide from a pronase digest of EDP208 pilin contains Asp, Thr, and Leu, in the order acetyl-NH-Thr-Asp-Leu. On the other hand, the N-terminal peptide from a pronase digest of ColB2 pilin contains six amino acids (Glu, Asp, Gly, Ala, and Leu [2:1:1:1:1]). Although the sequence of the ColB2 hexapeptide has not been determined, the ^{1}H NMR results indicate that the acetyl group is probably not on threonine.

The amino acid compositions of the soluble peptides generated from EDP208 pilin by pronase and trypsin digestion are very similar and one of the trypsin peptides probably contains the blocked N-terminal. However, sequence data on this peptide is not yet available because it has not yet been completely purified from the other trypsin-soluble peptides.

The remainder of each protein remains insoluble after pronase or trypsin digestion. This may represent one large insoluble peptide or several peptides. However,

SDS gel electrophoresis of the EDP208 insoluble material revealed only one band whose mobility could not be distinguished from that of the whole EDP208 pilin. Also, this insoluble material was subjected to gel filtration on Sephadex LH 60 columns using 88% formic acid:iso-propanol (30:70) as the solvent. The EDP208 insoluble fraction elutes as one sharp peak, suggesting, but not proving, that the EDP208 pronase and trypsin insoluble material is indeed one large peptide. This was still the case after the insoluble material was oxidized with performic acid as described in Materials and Methods.

DISCUSSION

At the initiation of this investigation, little was known about the physical and chemical properties of conjugative pili. Only preliminary results of the chemical characterization of F pili had been reported (Brinton, 1965; Beard *et al.*, 1972; Date *et al.*, 1977; Helmuth and Achtman, 1978). However, two other types of conjugative pili, EDP208 and ColB2, have now been purified and partially characterized.

These investigations revealed that EDP208 and ColB2 pili share many properties with those of F pili. All three types of pili are made up of pilin subunits whose molecular weights are between 10,000 and 12,000. Electron microscopic studies indicated that EDP208, F, and ColB2 pili are resistant to dissociation by denaturants such as urea, guanidine hydrochloride, and deoxycholate. Circular dichroism spectroscopy demonstrated that the three types of pilin possessed between 60-70% α-helical secondary structure.

When analysed chemically, the three pilin types were found to contain a high proportion of hydrophobic amino acids. Moreover, the amino acid composition of ColB2 and F pilin was very similar and significantly different from that of EDP208 pilin. Carbohydrate and phosphate analyses indicated that EDP208 and ColB2 pili possessed, like F pili, both phosphate and carbohydrate. This suggested that conjugative pilin was phosphorylated and glycosylated.

However, when EDP208 and ColB2 pilin was purified further by dissociation in SDS and column chromatography, differences between the two were discovered. This procedure removed all of the carbohydrate from EDP208 pilin, but failed to remove the phosphate. The same technique failed to remove any of the phosphate from ColB2 pilin but removed all of the carbohydrate except glucose. When EDP208 and ColB2 pilin was precipitated with acetone or extracted with chloroform-methanol, all of the phosphate was removed from EDP208 pilin and all but one phosphate was removed from ColB2 pilin. Moreover, acetone precipitation failed to remove glucose from ColB2 pilin. The pilus-associated phosphate was almost entirely accounted for as phosphatidylglycerol and phosphatidylethanolamine for both pilin types. Based on these findings, it was concluded that EDP208 pilin does not contain covalently bound phosphate or carbohydrate. On the other hand, ColB2 pilin may contain one covalently bound glucose and one covalently bound phosphate. However, it was also possible that these components were bound to ColB2 pilin by another interaction which was resistant to SDS and acetone precipitation.

When EDP208, F, and ColB2 pilins were subjected to automated sequence analysis, it was found that the three proteins had blocked N-termini. Blocked N-terminal peptides from EDP208 and ColB2 pilin have now been isolated and the blocking group has been identified as an acetyl moiety. The sequence of the N-terminal peptide of EDP208

pilin was found to be acetyl-NH-Thr-Asp-Leu. The sequence of the N-terminal peptide of ColB2 pilin was not determined, but it was probably not the same as that of EDP208 pilin.

One of the major remaining questions about the genetics of F is whether or not the structural gene for F pilin is *traA*. Although adequate evidence has been provided that this is the case (Minkley *et al.*, 1976), direct proof would be the matching of the amino acid sequence of F pilin with the *traA* gene nucleotide sequence. Based on the elucidation of the nature of the blocking group on EDP208 and ColB2 pilin, and due to the demonstrated similarity between the latter and F pilin, it may now be possible to determine the N-terminal sequence of F pilin for this purpose.

When EDP208 and ColB2 pilin was digested with pronase or trypsin, only a small number of peptides were released from the insoluble protein. It would therefore be of interest to see whether such treatment releases the same peptides from intact pili, and if so, whether this has any effect on pilus morphology and function.

Finally, the chemical characterization of conjugative pili has been hampered by their hydrophobic nature. However, innovative techniques have now been developed to overcome this difficulty with pilin and with other hydrophobic proteins, such as bacteriorhodopsin (Gerber *et al.*, 1979). These techniques rely heavily on the use of detergents (SDS, Triton X-100, and deoxycholate) to

solublize the proteins. Gel filtration chromatography and organic solvent systems have also been utilized to separate hydrophobic peptides. Preliminary studies have indicated that procedures such as these may be of use in the further characterization of conjugative pili.

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APPENDIX A

Calculation of apparent α-helical content of pilin from molar ellipticity.

The percent α-helical content of pilin was calculated by solving the following simultaneous equations using the experimental molar ellipticities at 215, 220, and 225 nm and the theoretical values for 100% α-helix, 100% β-sheet and 100% random coil, provided by Chen et al. (1972).

$$(1)\qquad \theta_{215} = -25.7\, f_{\alpha} - 9.34\, f_{\beta} + 0.669\, f_{RC}$$

$$(2)\qquad \theta_{220} = -29.5\, f_{\alpha} - 6.06\, f_{\beta} + 1.8\, f_{RC}$$

$$(3)\qquad \theta_{225} = -28.7\, f_{\alpha} + 1.54\, f_{\beta} + 0.264\, f_{RC}$$

Where θ_{λ} is the experimental molar ellipticity at the indicated wavelength and f_{α}, f_{β}, and f_{RC} represent the fraction of α-helical, β-sheet, and random coil structure in the protein.

The solution to these equations for f is:

$$(4)\qquad f = \frac{(-7.87\,\theta_{215} + 2.92\,\theta_{220}) + (-3.37\,\theta_{220} + 22.97\,\theta_{225})}{676.75}$$

This equation was used to calculate the % α-helix of pilin in the various denaturants.

APPENDIX B

Amino Acid Composition of N-terminal Peptides

from EDP208 and ColB2 Pilin[a]

Amino acid	Number of residues	
	EDP208 peptide	ColB2 peptide
Lys	0	0
His	0	0
Arg	0	0
Asp	0.97	0.96
Thr	1.04	0
Ser	$<$ 0.2	$<$ 0.5
Glu	$<$ 0.2	2.02
Gly	$<$ 0.2	1.18
Ala	0	1.04
½Cys	n.d.[b]	n.d.
Val	0	0
Met	0	$<$ 0.3
Ile	0	0
Leu	1.00	0.84
Tyr	0	0
Phe	0	0
Trp	-	n.d.
amount[c]	465 nmole	160 nmole

a. Isolated by passing the pronase-soluble peptides through a cation exchange column and eluting with distilled water as described in Materials and Methods.

b. Not determined.

c. This is the amount of each peptide cleaved from 500 nmole of EDP208 and 200 nmole of ColB2 pilin.